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Effect of Internalized Prebiotic Nanoparticles on the Anti-Pathogenic Activity of Probiotic *Pediococcus acidilactici*

생균제 ***Pediococcus acidilactici*** 의 항병원성 활성에 미치는

프리바이오틱스 나노입자의 도입 효과에 관한 연구

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농 학 박 사 학 위 논 문

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지도교수 최 윤 재

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Summary

Antibiotics have been used in many fields, such as medicine, food preservation, and the livestock industry. However, the increasing number of antibiotic resistant strains of bacteria has increased bacterial infections and threatens modern society. The World Health Organization (WHO) recently revealed that antibiotic resistant bacteria have reached alarming levels in many nations of the world. Therefore, in the livestock industry, using antibiotics in animal feed has been prohibited. Thus, developing alternatives to antibiotics has become important in the livestock industry. Probiotics as alternatives to antibiotics are an emerging field. Probiotics can confer health benefits to the host when the appropriate amounts are administered because they have their own antimicrobial properties and can manage the gut microorganisms. Among the many probiotics, *Pediococcus acidilactici* (PA) has been widely used in the animal and food industry. PA is known to have antimicrobial activity against many pathogens such as *Escherichia coli*, *Salmonella*, and *Listeria* because it produces antimicrobial molecules (lactic acid and bacteriocin) and modulates the gut system. However, there are still some limitations on using probiotics as alternatives to antibiotics due to their low production of antimicrobial substances.

Given that prebiotics stimulate the growth and/or activity of probiotics located in the digestive tract and confer favorable health effects on the host, many strategies have been selected to use prebiotics to raise the growth or activity of probiotics. Sometimes, inorganic nanoparticles have been used because they have

antimicrobial activity. In this study, prebiotics were chemically modified with hydrophobic groups to form prebiotic nanoparticles. To improve livestock productivity, as a replacement for antibiotics, prebiotic nanoparticles were used to enhance the antimicrobial ability of probiotics. Two types of polysaccharides, inulin and dextran, were used to develop the prebiotic nanoparticles. The prebiotic nanoparticles were prepared by conjugating hydrophobic groups such as phthalic anhydride, acetic anhydride, or propionic anhydride with polysaccharides. The effect of the different types of prebiotic nanoparticles on the cellular and antimicrobial activities of PA were investigated by in vitro and in vivo experiments.

In study 1, three types of inulin nanoparticles were developed. Phthalyl- (PINs), acethyl- (AINs) and propyl-inulin nanoparticles (PrINs) were developed as prebiotics, and their effects were observed on the cellular and antimicrobial activities of PA. The antimicrobial activities against pathogens were tested after the treatment with those nanoparticles for PA. All three types of inulin nanoparticles increased the antimicrobial property of PA. Among the three types of inulin nanoparticles, phthalyl inulin nanoparticles (PINs) showed the highest antimicrobial activity against pathogens. Therefore, a mechanism study was done to examine how the PINs could increase the antimicrobial property of PA. The internalization of the PINs into PA was first assessed. The internalization of the PINs was largely regulated by glucose transporters in PA, and the process was energy- and size-dependent. After the internalization of the PINs, a substantial

amount of antimicrobial peptide (pediocin) was produced by PA. Therefore, the higher amounts of pediocin could be more effective against both Gram-positive (*Salmonella* Gallinarum and ETEC K88) and Gram-negative (*L. monocytogenes*) pathogens than PA alone or PA treated with inulin. When treated with the smallest PINs, PA exhibited a nine-fold increase in antimicrobial activity. The rise in pediocin activity in PA treated with PINs was accompanied by the enhanced expression of stress response genes (*groEL*, *groES*, and *dnaK*) and pediocin biosynthesis genes (*pedA* and *pedD*). Overall, the results suggest that the internalization of the PINs by PA causes mild stress in PA activating the defense mechanism which leads to an increase of pediocin production.

In study 2, to demonstrate the previous concept with different types of prebiotic nanoparticles, phthalyl dextran nanoparticles (PDNs) were developed as prebiotics. Initially, the antimicrobial property of PA significantly increased after the treatment with PDNs. Moreover, there was no difference between the PINs and PDNs for enhancing the antimicrobial activity of PA. Therefore, to identify if the mechanism for increasing the antimicrobial property of PA with PDNs was same as the PINs, another experiment was conducted with the PDNs. The internalization of the PDNs by probiotics was dependent on temperature, time, and the glucose transporter. Internalization of the PDNs enhanced the production of an antimicrobial peptide which resulted in the higher antimicrobial properties of the probiotics against Gram-positive and Gram-negative pathogens compared to those of probiotics without any prebiotics. Moreover, internalization of the

PDNs increased the gene expression of pediocin through a probiotic self-defense mechanism. These results were consistent with the previous results for the PIN nanoparticles. Moreover, in study 2, an animal experiment was done to see if the higher pediocin production may also suppress pathogenic gut infection. One pathogen, EHEC O157:H7, was chosen to test the antimicrobial effect of PA with internalized PDNs in animals. Probiotics internalized with PDN was able to suppress pathogenic gut infections by decreasing pathogens and increasing some other bacteria species that are related with the prebiotics. Moreover, the population of the gut microbiome in the probiotics internalized with the PDN group was altered toward the other groups, especially when compared with the pathogen only treated group.

Results from study 1 and 2 indicate that prebiotic nanoparticles can be an intracellular stimulator regulating probiotic bacterial metabolism and suggest a new avenue for the probiotic modulation of antimicrobial peptides and their use in the potential treatment of many gut diseases. Therefore, as a replacement for antibiotics, enhancing antimicrobial ability using prebiotic nanoparticles will eventually improve livestock productivity.

Keywords: Prebiotics, Probiotics, Nanoparticles, Internalization, Antimicrobial peptide, Pediocin

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List of Abbreviations

AGPs: Antibiotic grwoth promoters

AINs: Acethyl inulin nanoparticles

AU: Arbitrary unit

CAP: Cellulose acetate phthalate

CFU: Colony forming unit

CLSM: Confocal laser scanning microscopy

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

DLS: Dynamic light scattering

DSS: Dextran sulfate sodium

EHEC O157:H7: O157:H7 Antigen-Positive Enterohemorrhagic *Escherichia coli*

ETEC K88: K88 Antigen-Positive enterotoxigenic *Escherichia coli*

ELS: Electrophoretic light scattering

ETEC: Enterotoxigenic Escherichia coli

FASC: Fluorescence-activated cell sorting

F/B ratio: Firmicutes/Bacteroidetes ratio

FITC: Fluorescein isothiocyanate

FPKM: Fragments per kilobase million

GI: Gastrointestinal

H-NMR: Nuclear magnetic resonance

HSP: Heat shock protein

IBD: Inflammatory bowel disease

INs: Inulin nanoparticles

LAB: Lactic acid bacteria

L. monocytogenes: *Listeria monocytogenes*

PA: *Pediococcus acidilactici* 175 (KCTC21088)

PDNs: Phthalyl dextran nanoparticles

PINs: Phthalyl inulin nanoparticles

PrINs: Propyl inulin nanoparticles

qRT-PCR: Quantitative real-time polymerase chain reaction

RAST: Rapid annotation using subsystem technology

ROS: Reactive oxygen species

RT-PCR: Quantitative real-time polymerase chain reaction

Salmonella Gallinarum: *Salmonella enterica* serovar Gallinarum

SCFA: Short chain fatty acid

SEM: Scanning electron microscope

TEM: Transmission electron microscopy

TNBS: 2,4,6-Trinitrobenzenesulfonic acid

TNF: Tumor necrosis factor

Introduction

Until now, antibiotics have been used in large quantities in many parts of society, including the livestock industries. However, due to the appearance of antibiotic resistance bacteria, this has reached critical levels around the world (WHO 2014). Using antimicrobial growth promoters (AGPs) has been banned in animal feeds since 2006 in EU and 2011 in the Republic of Korea. After the prohibition of using AGPs, the productivity ultimately decreased and other various problems have emerged in livestock industries (Wierup 2001). These problems have led to the increase in using more therapeutic antibiotics in livestock industries. Therefore, developing efficient alternatives to antibiotics is an important issue in livestock industries to solve these problems. Currently, there are not many safe and effective strategies; however, many researchers are considering probiotics and prebiotics to improve the growth performance of livestock animals by inhibiting pathogenic infection, modulating gut microbiota, and modulating the immune system.

Probiotics are live microorganisms that can confer health benefits to a host when an adequate amount is administered. Therefore, probiotics are generally recognized as safe and their use is growing as an alternative to antibiotics. Among many bacteria, lactic acid bacteria (LAB) are usually used as probiotic strains (Ljungh and Wadstrom 2006) because LAB produces antimicrobial substances (lactic acid and antimicrobial peptides) and modulates the health of the host. *Pediococci* are Gram-positive lactic acid bacteria and are widely used in humans

and animals. The *Pediococcus acidilactici* strain is one of the most popular strains among *Pediococcus* spp. because they produce a bacteriocin called pediocin (Chikindas et al. 1993). This strain can survive in various conditions and is known to colonize in the digestive tract and imparts many beneficial effects to the host. By producing lactic acid and pediocin, this can also prevent pathogenic gut infection such as *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* spp. (Daeschel and Klaenhammer 1985). Moreover, probiotics can help nutrition uptake and modulate gut microbiota improving growth performance.

On the other hand, the antimicrobial activity of probiotics against pathogens are not very effective as antibiotics due to their low production of antimicrobial substances (Kailasapathy and Chin 2000). Therefore, to overcome these limitations on using probiotics as a replacement for antibiotics, a number of strategies including biological, physical and chemical methods have emerged. Biological bioengineering methods are nowadays preferable because they can manipulate bacteria to produce antimicrobial compounds or to improve tolerance in many stressful situations by making recombinant bacteria (Mathipa and Thantsha 2017). The physiological method is mostly used to optimize the growth condition such as the pressure and temperature of probiotics to produce the highest antimicrobial peptides (Garsa et al. 2014). The chemical method is used as a delivery system for carrying bacteria safely towards digestive enzymes or as cultural supplements such as prebiotics (Amalaradjou and Bhunia 2012).

To enhance the growth and activity of probiotics, prebiotics are used. Prebiotics are generally defined as indigestible polysaccharides that stimulate the growth or activity of probiotics in the gastrointestinal tract (Holscher 2017). Generally, most prebiotics consist of carbohydrate compounds with a bond that cannot be digested by host enzymes (Flint et al. 2012). Among them, Inulin, dextran, pullulan, and galacto-oligosaccharide are known as prebiotics and are known to confer favorable health effects on hosts with probiotics (Muramatsu et al. 2012; Olano-Martin et al. 2000; Slavin 2013).

Nanoparticles are widely used in many industries including the biomedical, energy generation, biosensors, and cosmetics industries, etc. In biomedical applications, polymeric nanoparticles have been largely used because they are known to overcome the various biological barriers (Petros and DeSimone 2010). Moreover, polymeric nanoparticles have already been used as a delivery carrier for probiotics (Cavalheiro et al. 2015). Additionally, some nanoparticles including organic and inorganic nanoparticles are known to have antimicrobial properties by themselves (Lam et al. 2018). However, there are still some limitations on using nanoparticles as antimicrobial therapy because they have toxicity against beneficial bacteria as well as the hosts.

The aim of this study was to evaluate the effect of prebiotic nanoparticles on the cellular and antimicrobial activities of probiotics. Therefore, the probiotic bacterium *Pediococcus acidilactici* (PA) was treated with different types of prebiotic nanoparticles in study 1 and study 2 (Figure 1). The changes in the

antimicrobial activity of PA were analyzed as well as the mechanism of the prebiotic nanoparticles after the treatment. Moreover, the internalization of the prebiotic nanoparticles was analyzed for various conditions. Furthermore, the changes in the physiological and population of the gut microbiota in animals were analyzed after PA was treated with the prebiotic nanoparticles.

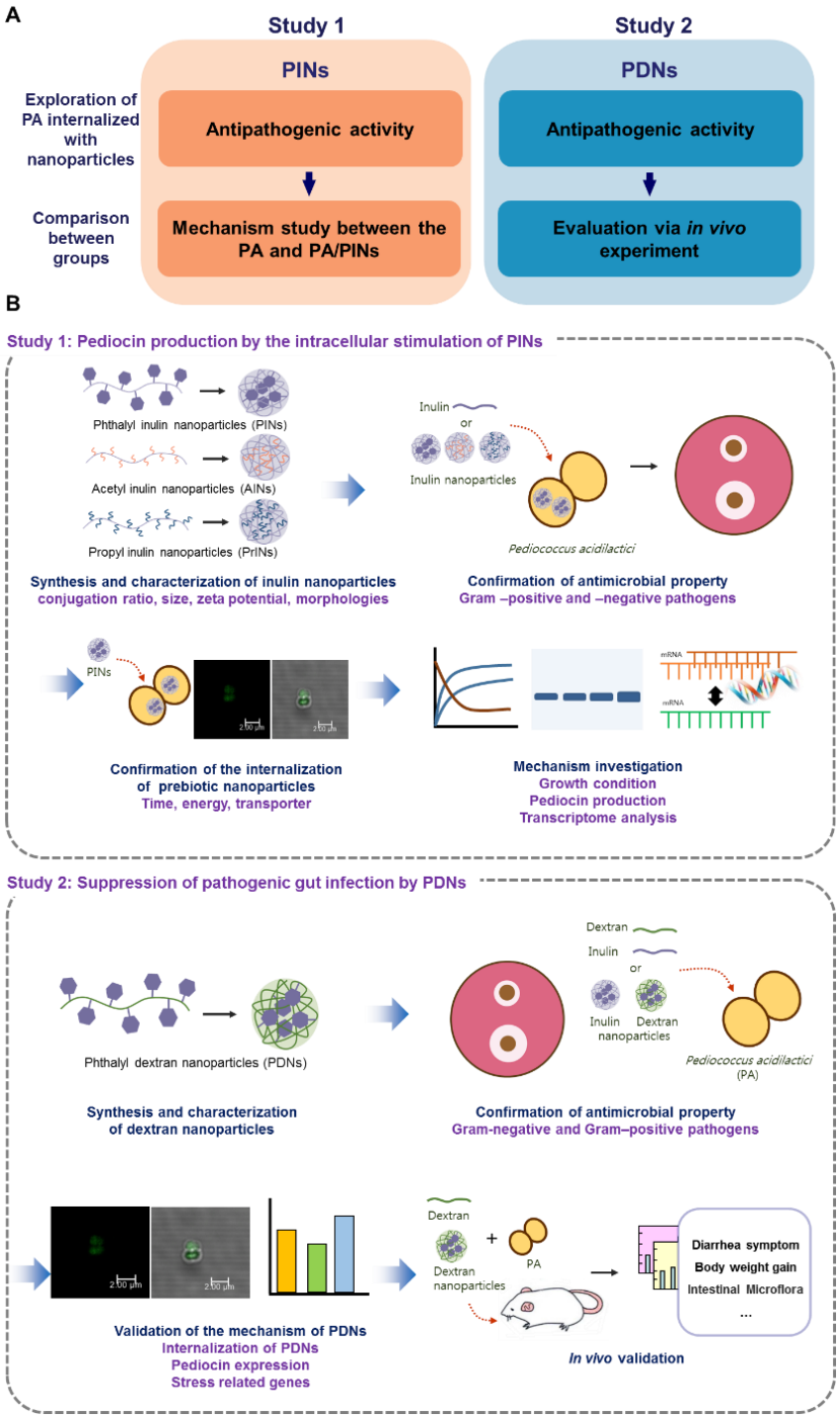


Figure 1. Aim of the study and experimental flow chart

Review of Literature

1. Necessity of alternative antibiotics

1) Antibiotics

Any medicines that kills or inhibits bacterial growth and used at a low dose is known as antibiotics (Soares et al. 2012). Antibiotics have led the world to a new era where many diseases can be controlled (Spellberg 2014). Not only are they used as medicine for humans, antibiotics are also used in many industries such as the livestock industry and food industry. In the livestock industries, antibiotics are used to control diseases and to promote growth. Therefore, people began to use antibiotics as feed additives for animal growth as animal growth promoters (AGPs).

Antibiotics can fall into two general categories, such as bacteriostatic and bactericidal. Most antibiotics used (99.9%) are bactericidal (Kohanski et al. 2007). Bactericidal antibiotics are known to kill bacteria directly whereas bacteriostatic antibiotics inhibits the growth of bacteria. However, in reality, distinction between the two classes are not sharp because bacteriostatic antibiotics also kill bacteria at a higher dose. The mechanism of antibiotics can be distinguished into three major categories (Kapoor et al. 2017). They can interfere with cell wall synthesis or maintenance, or nucleic acid synthesis, or protein synthesis (Figure 2). First, antibiotics such as beta-lactams (penicillins and cephalosporins), vancomycin,

bacitracin, and polymyxins, inhibit cell wall and membrane synthesis or function. Second, antibiotics inhibit nucleic acid synthesis or function by inhibiting DNA Gyrase (quinolones), or folate synthesis (trimethoprim, sulfamethoxazole), or create free radicals (metronidazole and nitrofurantoin). Lastly, there are some antibiotics that work on bacterial ribosomes and inhibit protein synthesis. Those that inhibit the 50S subunit are macrolides, streptogramins, and chloramphenicol whereas those ones that inhibit the 30S subunit include aminoglycosides, tetracyclines, and tigecycline.

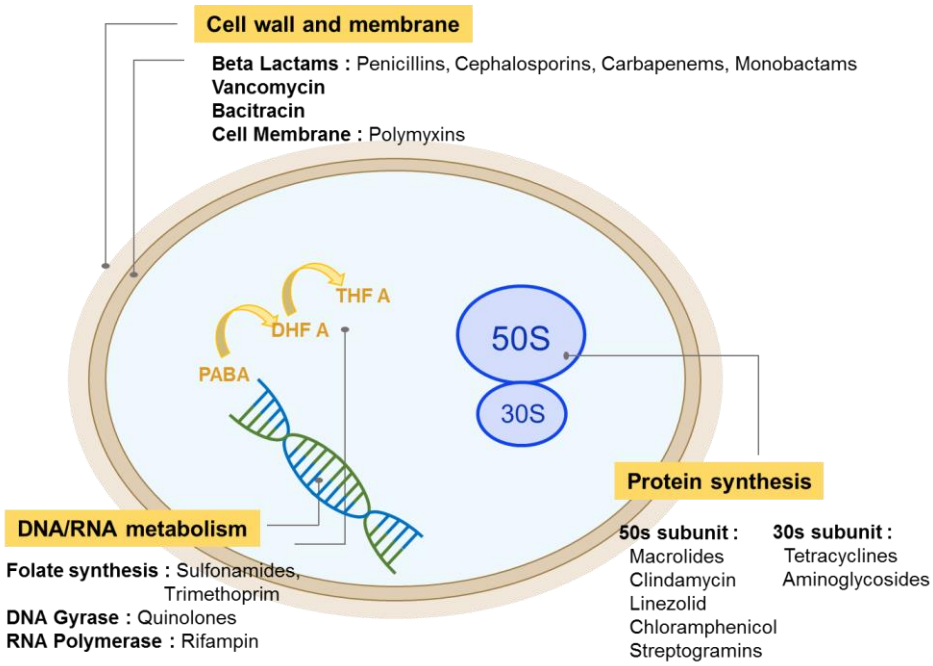


Figure 2. Antibiotic mechanisms of action. Antibiotics target various sites of the bacteria and inhibit the growth of the bacteria. The targets of antibiotics can be distinguished into three categories; (1) inhibiting cell wall and membrane permeability and synthesis, (2) DNA and RNA synthesis, and (3) protein synthesis

2) A need for alternative to antibiotics

Although AGPs as feed additives for livestock has improved the productivity, by the misuse and overuse of AGPs, antibiotic resistant bacteria, also known as super bacteria, have now reached a critical level (Figure 3A).

There are many mechanisms for now bacteria become resistant to antibiotics (Blair et al. 2015). Bacteria develop beta-lactamase to hydrolyze antibiotics such as penicillin. They also develop efflux pumps to expel drugs. These transport proteins are commonly known as multidrug resistance pumps. Moreover, bacteria alter the structure of antibiotics. For example, antibiotics that contain hydroxyl groups, such as chloramphenicol, can be acetylated by the enzymes produced by bacteria which inactivates the antibiotics (Kohanski et al. 2010). Bacteria can also alter their ribosomes or sequences. For example, folate synthesis is inhibited by some antibiotics (sulfonamide or trimethoprim); however, by the abuse of these antibiotics, bacteria transport folic acid from outside the cells into the cells rather than synthesizing it themselves (Ho and Juurlink 2011). Lastly, developing a bacterial biofilm is known to have antibiotic resistance due to their multicellular strategies.

By the introduction of antibiotic resistant bacteria, anxiety on using antibiotics for growth promotion became higher because antibiotics may accumulate in the tissue of animals (Ronquillo and Hernandez 2017). Therefore, in some countries AGPs have been banned (Figure 3B). However, the banning of AGPs has led to

the use of more therapeutic antibiotics. For these reasons, finding a replacement for antibiotics is needed.

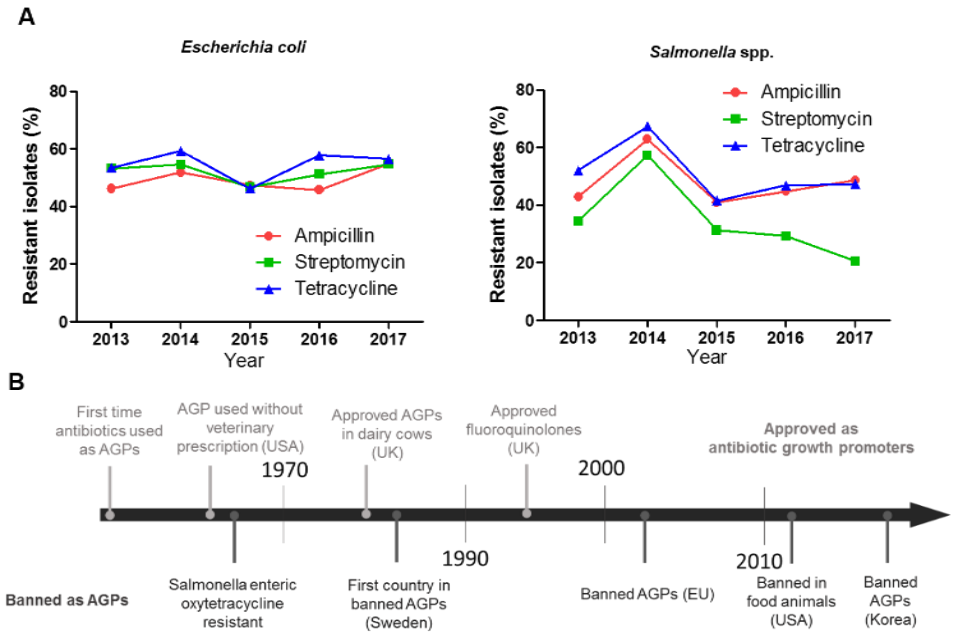


Figure 3. Resistant ratio and timeline of AGPs. (A) The total average resistant ratio of *E. coli* and *Salmonella* spp. in animals (pig, cow, and chicken) towards a different types of antibiotics. Animal and Plant Quarantine Agency, Korea (2013~2017) (B) Timeline of AGPs that were approved and banned. The figure is modified from Ronquillo and Hernandez (2017)

2. Probiotics

1) Definition of probiotics

Over the past few decades, there has been growing interest in the use of probiotics as potential alternatives for synthetic antibiotics and anti-inflammatory drugs, not only due to the side effects of synthetic drugs but also to the improper use of antibiotics that has promoted the development of antibiotic-resistant bacteria. The FAO/WHO defined probiotics as living microorganisms that inhibit pathogen growth and increase beneficial bacteria in the host gastrointestinal tract, which confers a health benefit to the host (FAO/WHO 2001). Various microorganisms are known as probiotics although most typical probiotics are the lactic acid bacteria (LAB), bacilli and yeast.

The majority of bacteria used as probiotics is LAB. They are Gram-positive bacteria and produce lactic acid (Von Wright 2011). In LAB, there are several genera; *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Sporolactobacillus*, *Oenococcus*, *Tetragenococcus*, *Weissella*, and *Vagococcus*. Moreover, there are probiotics that do not produce lactic acid. The *Bifidobacterium* species, *Escherichia coli nissle*, and *Saccharomyces cerevisiae* are two of them. These probiotics are currently used in fermented foods, drinks, cosmetics, medicines, and animal feed additives.

2) Characteristics of probiotics

Probiotics confer a health benefit to the host through various mechanisms. Generally, the effects of probiotics as a replacement for antibiotics can be classified into three modes of action (Figure 4). First, probiotics have a direct effect on pathogens by producing antimicrobial substances such as bacteriocins (Prabhurajeshwar and Chandrakanth 2017). Next, probiotics can modulate host defenses including the innate and acquired immune system. Lastly, probiotics affect the function of the host gut barrier (Oelschlaeger 2010; Yan and Polk 2011). Probiotics have been widely used in food and as feed additives due to their effects on the host such as nutrition absorption and changing the gut microflora.

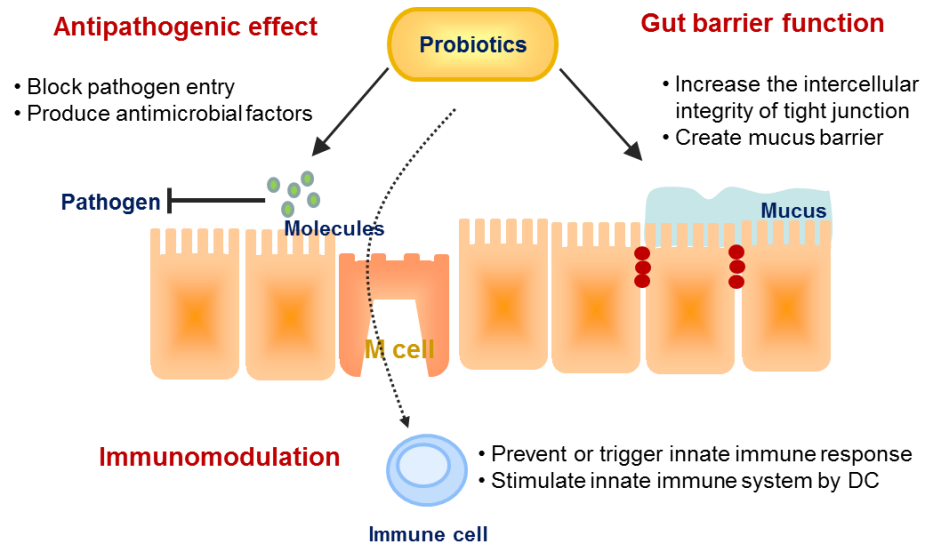


Figure 4. Biological effects and mechanism of probiotics. There are three major biological effects: antimicrobial effect, enhancing the function of the gut barrier, and modulating the host immune system.

(1) Antimicrobial properties against pathogens

The interaction between intestinal microbiomes are very complex, and inhibiting pathogenic bacteria in the intestinal tract is important to maintain the health of the host (Nagpal et al. 2012). Microorganisms that inhibits the growth of pathogenic bacteria by producing antimicrobial substances are known as probiotics. Lactic acid bacteria, major probiotics, produce lactic acid, bacteriocin, hydrogen peroxide, and citric acid as antimicrobial substances. *Bifidobacteria* produce acetic acid and lactic acid as antimicrobial substances. These inhibitory substances have been shown to be protective against pathogens including *E. coli*, *Salmonella*, *Listeria*, *Clostridium* and *Campylobacter* (Gareau et al. 2010).

Several LAB strains are already known to reduce the pathogenic infection level in various animals, and among them, *Pediococcus acidilactici* is known to protect against coccidiosis (Lee et al. 2007). *Lactobacillus plantarum* has shown a beneficial effect on IBD models in mice and diarrhea associated with antibiotics in humans (Brashears et al. 2003; Lonnermark et al. 2010). *Bacillus polyfermenticus* was effective against colitis induced by both DSS and TNBS (Im et al. 2009). *Bifidobacteria bifidum* could reduce necrotizing enterocolitis in a rat model (Khailova et al. 2009). Moreover, *Escherichia coli* Nissle 1917, a Gram-negative bacterium, is known to be beneficial in a colitis model that is induced by DSS (Ukena et al. 2007). Through these antimicrobial properties of probiotics, they can protect hosts from a variety of pathogens and also can function as alternatives to antibiotics.

(2) Immune modulation

There have been several reports that probiotics can influence the immune system of the host and reduce inflammation and diseases (Klaenhammer et al. 2012). Some probiotic strains such as *B. longum, B. thermophiles, L. acidophilus, L. casei subsp. rhamnosum, L. helveticus,* and *E. faecium* have been identified to have the property of increasing the immune response of host (Isolauri 2001; Ochoa-Reparaz et al. 2010; Rajilic-Stojanovic et al. 2015). Their metabolites, such as cell wall components and DNA, can be recognized by the host cells, including gut epithelial or gut-associated immune cells, and influence the immune system (Oelschlaeger 2010). The adhesion of probiotics to host cells triggers the immune signal. However, most bacteria cannot reach the epithelial cell because of the mucus layer (Matsuo et al. 1997). However, through the uptake and transcytosis of bacteria through M cells, probiotics can directly interact with the host immune system. Luminal gut antigens and dendritic cells are located in the lower level of M cells. Another direct interaction, besides M cells, is through dendritic cells. They uptake bacteria and presents the bacteria as antigens (Macpherson and Uhr 2004). Therefore, after the interaction with host cells, probiotics are able to enhance the immune system by increasing the secretion of immunoglobulin, the proliferation of lymphocytes, the production of interleukins 1, 2 and 6 and TNF and prostaglandin E, and increase the serum total protein, albumin, globulin, and gamma interferon (Nagpal et al. 2012).

Therefore, probiotics have the potential to control antibiotic-associated diarrhea, allergic responses, Crohn's disease, and ulcerative colitis. Moreover, because the immune function decreases with age, probiotics can be therapeutically used to enhance the immunity of the host (Gill and Rutherfurd 2001).

(3) Enhancement of host barrier function

To prevent infection from pathogens, maintenance of the gut epithelial barrier and function is another important issue. Probiotics can maintain the function of the intestinal epithelial barrier and can make the cell-cell junctions of the gut barrier tighter. Probiotics secrete many beneficial metabolites or molecules to regulate the tight junction integrity of the gut. The production of SCFA is one of the most popular mechanisms to enhance the function of the gut epithelial barrier (Rao and Samak 2013). Microorganisms in the digestive tract stimulate intestinal fermentation including a higher production of SCFA. These fatty acids can regulate the expression of the tight junction proteins in the intestinal cells. LAB are known to produce various enzymes and molecules, and mostly they produce lactate. The increased level of lactate itself in the intestine also can be an effective treatment in bowel syndrome and dyspeptic diarrhea patients (Tsukahara and Ushida 2001). However, there is no evidence yet that lactate can upregulate the function of the intestinal barrier. Mostly by lactate-utilizing bacteria, such as

Clostridia cluster XIVa, they can metabolize lactate into SCFA including acetate, propionate, and butyrate (Belenguer et al. 2006).

Bifidobacteria spp. are also effective in regulating the permeability of the gut. Supplementation with *Bifidobacteria* improved the intestinal integrity in DSS-induced colitis (Wang et al. 2006). Additionally, a mixture of *Lactobacillus* and *Bifidobacteria* was shown to be protective against acute colitis by upregulating the tight junction proteins occludin, ZO-1, and claudins (Mennigen et al. 2009). Though these LAB, *Bifidobacteria*, and lactate-utilizing bacteria, the concentration of SCFA increases in the digestive tract, and this helps to maintain an appropriate pH in the intestinal lumen and contributes as an energy source for the host cells to increase the intestinal integrity (Lawson 1990).

(4) Host metabolism

Several studies have shown that probiotics are associated with metabolic disease. Probiotics are known to affect the lipid metabolism of the host. Coronary heart and cardiovascular diseases are a major cause of death in human adults, and a sufficient therapy to reduce these diseases is to reduce the blood cholesterol levels (Nagpal et al. 2012). Supplementation of probiotics, including *L. acidophilus* and *Bifidobacterium* spp. and the intake of fermented milk or yogurt, have been shown to reduce the blood cholesterol levels and lower cholesterolemia in humans (Grunewald 1982; Mann 1977). The possible mechanisms of probiotics

in lowering cholesterol levels is to assimilate cholesterols, by deconjugation of bile salts, binding cholesterol to bacterial cell walls, and reducing cholesterol biosynthesis (Nagpal et al. 2012; Pereira and Gibson 2002).

Moreover, probiotics are known to reduce glucose levels and insulin resistance in diabetic and obese patients. It is now well documented that obesity and insulin resistance regulate the proportion of microflora of *Firmicutes* and *Bacteroidetes* (Ley et al. 2005). Mostly, it is reported that obesity groups have a higher F/B ratio than that of lean groups; however, there are still some controversial results (Clarke et al. 2014; Delzenne and Cani 2011; Kasai et al. 2015). Therefore, modulating the gut microflora could be one of the targets to manage obesity and diabetes. Because probiotics modulate gut microflora, supplementation of probiotics can prevent obesity and diabetes. Moreover, specific strains such as *Lactobacillus* spp. and *Bifidobacteria* spp. have been shown to decrease body weight gain or insulin resistance in humans (Kang et al. 2010; Naito et al. 2011). Probiotics and gut microbiota also increase energy harvest and lower inflammation; thus, these may manage obesity and diabetes (Delzenne et al. 2011). Hence, modulating gut microflora based on the supplementation of probiotics can be a potential therapy against obesity and diabetes.

(5) Regulation of the gut microbiota composition

Supplementation of probiotics is known to affect the composition of the intestinal microbiota in the digestive tract. In the animal gut, a variety of different microorganisms live in the gastro-intestinal tract, including bacteria, archaea, fungi, protozoa, and viruses (Sekirov et al. 2010). Through the development of next-generation sequencing, the number of gut microorganisms existing in the gut was found to be about 10^{14} microbes including 500 to 1,000 species of bacteria (Gill et al. 2006).

The role of the microbiota in the gut system has garnered much attention in recent years because they interact mutually with the host, depending upon their composition and communication, and affect our health in terms of cancer, aging, obesity, immune system, and antimicrobial activity (Buffie and Pamer 2013; Hooper et al. 2012; Louis et al. 2014; O'Toole and Jeffery 2015; Shen et al. 2013) (Figure 5). After birth, microorganisms colonize rapidly in the gastro-intestinal tract. In the early stage, the major phyla are *Actinobacteri*a and *Proteobacteria* (Rodriguez et al. 2015). As the gut microbiota grow, its profile changes, and in healthy individuals, the major phyla are *Proteobacteria* and *Bacteroidetes*, which are Gram-negative bacteria and for Gram-positive bacteria, *Firmicutes* are the predominant archaea in the gut (Dridi et al. 2011). In older individuals, the gut microbiota is relatively stable, however, the composition can be altered by the host diet, antibiotic treatment, infection, inflammation, and stress. Therefore, to alter the gut microbiota into a healthy state, supplementing probiotics or prebiotics are

one of the strategies. For example, one study supplementing a mixture of *Lactobacilli* and *Bifidobacteria* has shown that pathogenic gut infection can be prevented in animal models (Kondepudi et al. 2014). Furthermore, in IBD patients, modulating the gut microbiota is now considered as a therapy. In summary, probiotics are now used to confer health benefits to the host by the administration of adequate amounts (Hill et al. 2014).

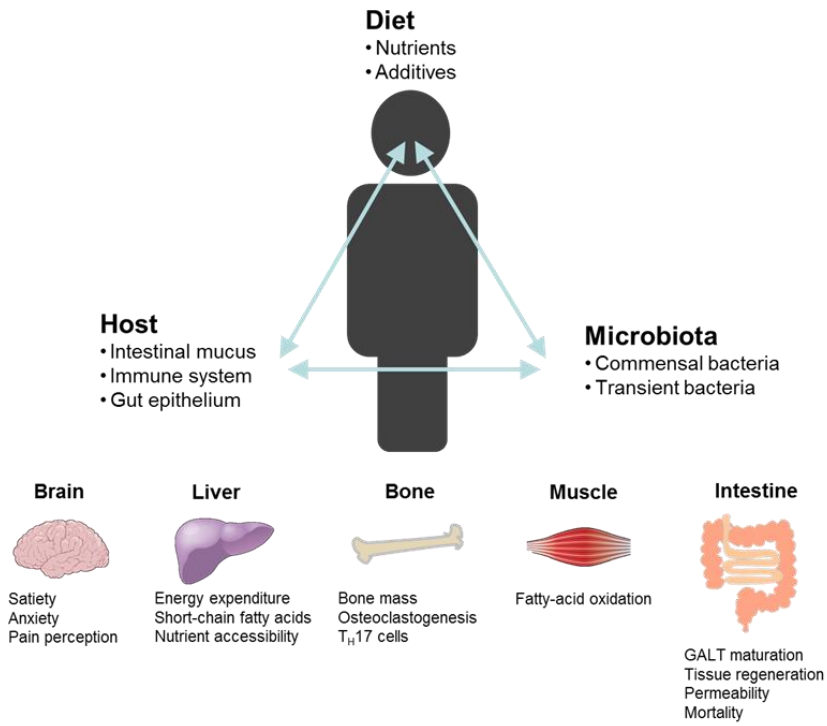


Figure 5. Relationship between gut microbiota and host physiology. The gut microbiota composition can be influenced through diets or the host immune system. The gut microbiota may influence the host physiology by influencing the process in various organs. The figure is modified from Tremaroli and Backhed (2012).

3) *Pediococcus* as probiotics

(1) *Pediococcus* spp.

Pediococcus is a genus of Gram-positive lactic acid bacteria, belonging to the family of *Lactobacillaceae*. So far, the genus *Pediococcus* spp. is composed of *P. acidilactici, P. pentosaceus, P. inopinatus, P. dextrinicus, P. damnosus, P. parvulus, P. halophilus* and *P. urinaeequi* (Porto et al. 2017). They usually have a spherical shape and are found in pairs or tetrads as the other lactic acid cocci genera.

Many strains can differ in their tolerance to pH, temperature, oxygen, and osmolarities (Nout 1994). They are homofermentative, even though the assimilation and fermentation features of carbohydrates may differ among the strains and species. Research on the *Pediococcus* genus is continuously growing, especially with *P. acidilactici* and *P. pentosaceus*. Because *P. acidilactici* and *P. pentosaceus* have the ability to produce bacteriocin, this phenomenon enabled them to be used as biopreservatives in food products and as a replacement for antibiotics (Papagianni and Anastasiadou 2009).

(2) *Pediococcus acidilactici*

Pediococcus acidilactici is a Gram-positive bacteria, known to grow in a wide range of pH, temperature, and osmotic pressure (Porto et al. 2017). *P. acidilactici* is catalase negative, and oxidase negative and able to ferment glucose, fructose, mannose, and galactose (Porto et al. 2017). They can survive in gastric conditions

for 3 h and have resistance to bile salts for 48 h. Moreover, *P. acidilactici* is known to colonize in the digestive tract and confers health benefits to the host (Klaenhammer 1993). In addition, they can produce lactic acid and bacteriocin called pediocin, which can inhibit the growth of enteric pathogens.

(3) Secreted molecules

Probiotics release small peptides or metabolites to defend against pathogens and to grow in restricted environments (Lebeer et al. 2008). Therefore, developing strategies that enhance the production of these small peptides or metabolites that have an antimicrobial action or modulating action on immune responses is needed.

① **Organic acids** LAB are known to produce organic acids such as lactic, acetic, and citric acids that are generally recognized as safe. Among them, lactic acid is the most effective organic compound to control the growth of microorganisms (Sirsat et al. 2009). Lactic acid is produced by LAB by fermenting simple carbohydrates such as glucose, galactose, and sucrose. Because organic compounds including lactic acid reduce the environmental pH. They have the capacity to inhibit microbes or decompose biological materials that cannot live in low pHs, including many enteric pathogens. This has been demonstrated in many studies that LAB, including *Pediococcus* strains, can inhibit the growth of pathogens *in vitro* and *in vivo* (Bajpai et al. 2016; Bartkiene et al. 2016; Helander et al. 1997). Therefore, based on this biological effect, lactic acid has been

approved as a food additive in the EU, USA, and Australia, and in New Zealand as a food preservative and flavoring agent (Anonymous 2011; FDA 2011; Legislation 2011).

LAB are also known to produce hydrogen peroxide and to create a nonspecific barrier as an antimicrobial activity (Pridmore et al. 2008). Additionally, hydrogen peroxide is used in combination with organic acids to enhance their antimicrobial activities (Lin et al. 2002).

② **SCFA** Probiotics including *Pediococcus acidilactici* produces SCFAs such as acetate, propionate, and butyrate (Ceapa et al. 2013). They directly inhibit pathogens and modify the microbial composition (Dobson et al. 2012; Hassan et al. 2012). As lactic acid, acetic acid reduces intestinal pH resulting in the inhibition of growth of some pathogens like *Salmonella* and *E. coli*. Propionate and Butyrate work as an energy source for epithelial cells in the gut and induce antimicrobial peptide expression in host cells (Vrieze et al. 2012). For example, SCFAs produced by probiotics can disrupt the outer membrane of Gram-negative pathogens and decrease the infection of pathogens such as EHEC O157:H7, *S. Typhimurium*, and *Pseudomonas aeruginosa* (Amalaradjou and Bhunia 2012; Carey et al. 2008; Fayol-Messaoudi et al. 2005).

③ **Bacteriocin** Bacteriocins are antimicrobial peptides that are synthesized in the ribosomes by Gram-positive and Gram-negative bacteria. They act as a bacteriocidal and bacteriostatic antibiotics on other bacteria. Mostly, bacteriocins that are produced by Gram-positive bacteria are known to be more effective against closely related bacteria. However, Gram-negative organisms (such as *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Salmonella*, *Shigella*, and *Pseudomonas*) are known to be sensitive to bacteriocins that are produced by Gram-positive ones after inflicting sublethal injuries like exposure to lactic acid, or hydrostatic pressure (Rodriguez et al. 2002). Various bacteriocins are produced by different bacteria (Prudencio et al. 2015) (Table 1). Bacteriocins can vary based on various conditions causing variation in their antimicrobial activity. The culture pH, temperature, and fermentation volume can also affect the antimicrobial properties of bacteriocin. Additionally, many enzymes such as proteinase K, lipase, alpha-amylase, and catalase can also change their antimicrobial activity (Rodriguez et al. 2002).

Table 1. Various types of bacteriocins and antimicrobial compounds

Probiotics	Compound
Pediococcus acidilactici	Pediocin PA-1, AcH, SJ-1
Pediococcus pentosaceous	Pediocin
Lactobacillus GG	Wide-spectrum antibiotic
Lactobacillus acidophilus	Acidolin Acidophilin Lactocidin Acidocin
Lactobacillus plantarum	Lactolin Plantaricin EF, JK, S, A
Lactobacillus reuteri	Reuterin Reutericylin
Lactobacillus gasseri	Gassericin A
Lactobacillus salivarius	Salivaricin A
Lactococcus lactis	Nisin A, Z Lacticin 3147 Lactostrepsin Lactocin
Enterococcus faecalis	Cytolysin
Enterococcus faecium	Entericin A, P
Bacillus spp	Mersacidin

④ **Pediocin** Among the various bacteriocins, pediocins are a bacteriocin that are produced by *Pediococcus* spp. *Pediococcus acidilactici* strains. They produce pediocin PA-1, AcH, and SJ-1. These pediocins belong to bacteriocin class 2a, characterized as small unmodified cationic peptides (3.5~8 KDa) containing about 44 amino acid residues (Rodriguez et al. 2002). They have a conserved region called a pediocin box in the N-terminal and diverse hydrophobic or amphiphilic regions in the C-terminal that are responsible for cell recognition (Porto et al. 2017).

Pediocin has the potential to permeabilize the bacterial cell membrane and transports out cytoplasmic molecules to the outside of the cells eventually causing apoptosis in the cells. The helical domain at the pediocin N-terminal region binds to membrane receptors by electrostatic force. The poration complex is formed after the binding of pediocin, and this leads to cytoplasmic molecules moving to the outside of the cell (Porto et al. 2017) (Figure 6).

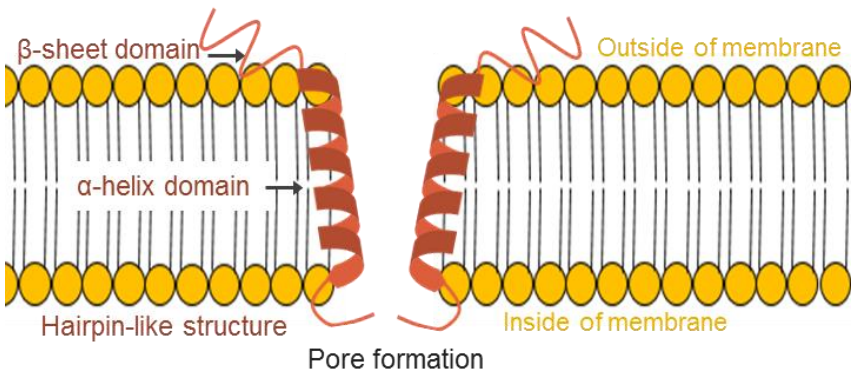


Figure 6. Operating principle of the bacteriocins

Pediocin is biosynthesized by four DNA fragments (*pedA, pedB, pedC,* and *pedD*), and the induction factor of pediocin is a bacteriocin-like peptide with a double glycine leader without a bacteriocin activity (Nes et al. 1996; Rodriguez et al. 2002). The *pedA* gene is called prepediocin, which consists of a double-glycine leader sequence and has the same biological activity as bacteriocin(Ray et al. 1999; Venema et al. 1995).

The *pedB* gene acts as an immunity protein which protects the producing cells from their own pediocin. The *pedC* gene works as an accessory protein that is involved with ABC transporters. The *pedD* gene encodes PedD, an ABC transporter that functions during pediocin secretion in cells. The proteolytic domain of PedD binds to prepediocin and removes the leader sequence to form the mature pediocin. The removed double-glycine leader sequence from *pedA* may act as an induction factor (Barrangou et al. 2006) which has been reported to represent a quorum-sensing phenomenon (Kleerebezem et al. 1997) (Figure 7).

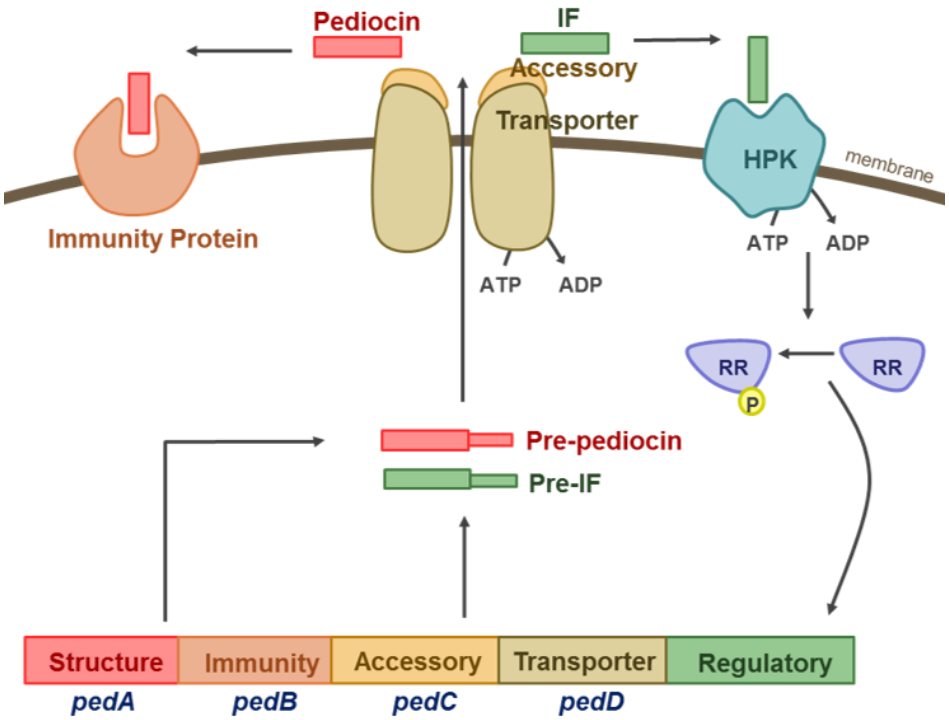


Figure 7. Bacteriocin biosynthesis process. Pediocin is produced through the interaction of four biosynthesis genes, *pedA*, *B*, *C*, and *D*. Regulatory genes produce bacteriocin-like protein to work as the induction factor of pediocin.

⑤ Heat shock proteins Heat shock proteins are proteins that help polypeptide from unfolding or misfolding, especially under stress conditions (temperature, pH, osmotic stress) (Zugel and Kaufmann 1999). In non-stressed conditions, low level of heat shock proteins are presented, however, when the cells are stressed, high level of heat shock proteins are produced (Zugel and Kaufmann 1999). The function of the heat shock proteins are mainly a molecular chaperone which is a critical role in protein folding; moreover, they can also modulate immune systems of the host (Binder 2014; Walter and Buchner 2002). In eukaryotic cells, heat shock proteins are named as HSP60, 70, and 90, etc by their molecular weight. In prokaryotic cells, heat shock proteins are named differently to eukaryotic cells such as GroES and EL, DnaK and J, and ClpB, etc (Table 2).

GroEL consists two rings each formed by seven identical protein subunits and GroES is a single-ring heptamer that acts together with GroEL in presence of ATP (Martin et al. 1993; Schlesinger 1990). They both serve in protein folding and assembling. DnaK, DnaJ, and GrpE are co-chaperones which work together in protein folding. DnaK hydrolysis ATP and binds to newly synthesized unfolded polypeptide chains, which help their folding without aggregations (Zolkiewski 1999). ClpB also cooperates with DnaK, DnaJ, and GrpE. ClpB has ATPase activity, which acts before the DnaK. By hydrolysis of ATP, protein is denatured and the hydrophobic binding sites may expose and help DnaK to refold the denatured proteins.

In addition, by any signs of stress, production of heat shock proteins gets higher and these may lead to a higher production of bacteriocins. Plantaricin, the bacteriocin produced by the *L. plantarum*, presents differently according to the different growth and stress conditions (Daranas et al. 2018; Sabo et al. 2014). Moreover, nutritional stress such as with magnesium or manganese affected the production of bacteriocins (Martinez et al. 2013).

Table 2. Classification of heat shock proteins

Approximate molecular weight (kDa)	Proteins	Function
10	GroES	Co-factor of GroEL
20-30	GrpE	Nucleotide exchange factor for DnaK
40	DnaJ	Co-factor of Hsp70(DnaK)
60	GroEL	Provides proper folding of proteins in presence of ATP
70	DnaK	Involved and prevents in protein folding and unfolding, gives thermotolerance to cell
100	ClpB	Provides tolerance to cell at extreme temperature Involves in protein folding

(4) *Pediococcus* spp. as probiotics

Many studies have shown that supplementation of probiotic bacteria in animal feeds increases growth performance of livestock animals and enhances the quality of the livestock products such as milk, eggs, and meats. Moreover, they can prevent diseases and improve feed intake, body weight, feed conversion ratio, and average daily gain in livestock animals (Di Giancamillo et al. 2008; Frizzo et al. 2008).

LAB including *Pediococcus acidilactici* are commonly used as probiotics (Di Giancamillo et al. 2008). Supplementation of *Pediococcus acidilactici* with LAB in a mouse diet decreased the presence of pathogenic bacteria such as *Staphylococcus* spp., *Enterococcus* spp., and *Clostridium* spp., in mice (Umu et al. 2016). Additionally, they increased the proportion of LAB in the animal gut. This result was consistent in most studies, *Pediococcus acidilactici* was able to decrease the infection level of *L .monocytogenes* in the animal gut (Fernandez et al. 2016). Moreover, *Pediococcus acidilactici* was able to decrease hepatic cholesterol and triglyceride levels in obese mice (Moon et al. 2014).

In addition, *Pediococcus acidilactici* is also used in many livestock and food products. They are used in milk fermentation and in sausages and shellfish such as oyster, mussels, and clams (Papagianni and Anastasiadou 2009).

4) limitations of conventional probiotics

Even though there are many advantages and health benefits provided by probiotic or probiotic food products, there are still certain limitations. Some studies have shown that probiotics may be inefficient or ineffective for some pathogens (Mathipa and Thantsha 2017). Probiotics release antimicrobial compounds; however, there are still some limitations in inhibiting pathogens with their own antimicrobial compounds. For example, LAB including *Lactobacillus* could not prevent pathogens by itself pathogens such as *L. monocytogenes*, from adhering and colonizing (Koo et al. 2012). Therefore, nowadays, a combination of various probiotic strains are used to enhance the effect against pathogens (Kailasapathy and Chin 2000).

Additionally, probiotics are known to modulate the immune system of hosts, however, the immune response may be too low or high in *in vivo* situations (Jankovic et al. 2010). Moreover, the activity of probiotics can change by their interactions with other bacteria, and this may result in a lower reproducibility (Karimi and Pena 2008). To overcome these situations and to enhance the activity of probiotics, more novel and innovative approaches should be found. Thus, novel strategies using biological, physical, chemical, and other techniques enable the increase in probiotic functions (Mathipa and Thantsha 2017).

3. Strategies to enhance antimicrobial activity of probiotics

A number of strategies have emerged to enhance the activity of probiotics such as the production of antimicrobial substances, resistance to stress tolerance and/ or preventing colonization of pathogens with biological, physical, and chemical methods (Figure 8).

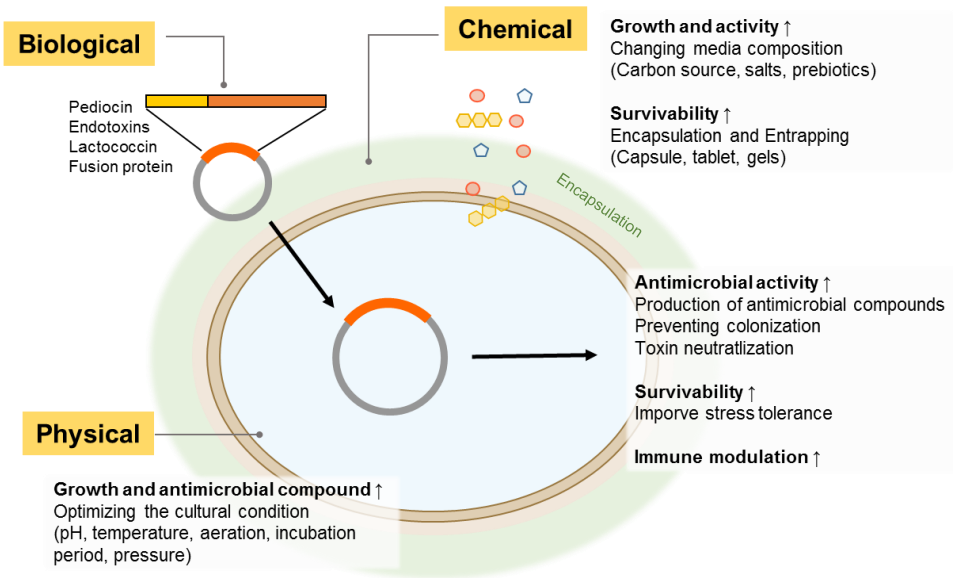


Figure 8. Strategies to enhance activity of probiotics. Modulating the properties of probiotics can be distinguished into three categories; biological modification, optimizing physical culture conditions, and treatment with chemical compounds or encapsulation

1) Biological method

Biological engineering strategies may propel probiotics to yield antimicrobial peptides with greater stability and enhanced features (Papagianni and Anastasiadou 2009). The manipulation of the genes of probiotic strains not only can improve the production of antimicrobial compounds but also can improve tolerance to stress, immune responses, or toxins. These improved features may help probiotics to survive or colonize better in the digestive tract to confer health benefits to hosts (Aditya Upadrasta 2011). Improving different functional properties of probiotics with recombinant techniques will inhibit the infections of pathogens and clinical symptoms (Table 3).

(1) Production of antimicrobial compounds

Some probiotics produce antimicrobial compounds or peptides as a defense mechanism against pathogens (Amalaradjou and Bhunia 2013). These antimicrobial compounds or peptides are known to be effective against antibiotic resistant pathogens because the mechanism of these compounds does not lead to resistance.

Some researchers have designed various microorganisms to express various antimicrobial peptides in many different probiotics. The antimicrobial peptide, which is produced by *Pediococcus acidilactici*, was expressed in a bactericidal yeast strain (*Saccharomyces cerevisiae*) (Schoeman et al. 1999). Among the pediocin biosynthesis genes, the *pedA* gene was successfully inserted and secreted.

The secreted pediocin peptide size was almost the same as the natural pediocin, approximately 4.6 KDa. The secreted peptide could inhibit the growth of *L. monocytogenes* B73. Therefore, they were able to conclude that the secreted peptide from yeast could biologically act as an antimicrobial peptide. *L. lactis* was designed to produce an antimicrobial peptide (A3APO and alyteserin) which was effective against Gram-negative pathogens in both *E. coli* and *Salmonella* strains, and the viability of *L. lactis* was maintained (Volzing et al. 2013). *L. casei* was engineered to produce the human antimicrobial peptide lactoferrin (Chen et al. 2010). The secreted lactoferrin successfully acted as an antimicrobial peptide against enteric pathogens.

Moreover, a technique also used is the production of efficiently fused antimicrobial peptides using the native genes that are already found in the host bacteria. To increase the production yield of an antimicrobial peptide, Beaulieu et al. fused the thioredoxin gene to the pediocin gene (*PedA*) and produced it in *Escherichia coli* (Beaulieu et al. 2007). PedA did not show any biological activity as an antimicrobial peptide with thioredoxin fused. However, after the cleavage of thioredoxin, the characteristics (molecular mass, biological activity and physiochemical properties) of the recombinant pediocin PA-1 were similar to those of the native pediocin. In addition, the production yield was almost 4 to 5 fold higher than the natural *Pediococcus acidilactici* PAC 1.0. Another study had fused a mature pediocin with the leader sequence of lactococcin A, naturally produced by *Lactococcus lactic* IL1403, to produce a higher yield of pediocin

(Horn et al. 1999). It was successfully expressed in *L. lactis* IL1403. Additionally, when the pediocin PA-1 gene was introduced to the *L. lactis* F15876 strain, already known to produce nisin, both nisin and pediocin PA-1 were produced simultaneously. Additionally, due to the characteristics that bacteriocins are produced by the quorum sensing system, a bacteriocin producing *E. coli* was engineered to express the LasR protein (Goh et al. 2012). The LasR protein is originally known to be released by *Psedomonas aeruginosa* to detect homoserin lactone and controls the expression of virulence genes. By producing the LasR protein in *E. coli*, this could increase the production of bacteriocin and inhibit *Psedomonas aeruginosa* growth and the formation of biofilms.

However, until now the production of antimicrobial peptides in probiotics is still expensive and time-consuming. Moreover, the peptides can kill the probiotics or producing cells and can be secreted as inclusion bodies which may have no functionality.

(2) Preventing colonization and toxin neutralization

Enhancing probiotic adhesion to the intestinal mucus or expressing pathogenic toxin receptor binding sites in probiotics may decrease and interfere with pathogen infections. A number of studies have already used these ideas to manage pathogens. The binding ability of *L. lactis* to human epithelial cells was enhanced after engineering *L. lactis* to express internalin A from *L. monocytogenes*

(Innocentin et al. 2009). Moreover, expressing the surface associated flagellin of *B. cereus* in *L. lactis* enhanced adhesion. This recombinant probiotic inhibited the binding of pathogenic *E. coli* and *S. enterica (Sanchez et al. 2011)*. To inhibit the adhesion of *Listeria*, *Listeria* adhesion protein (LAP) was expressed in *L. paracasei (Koo et al. 2012)*. LAP is known to interact with the host cell receptor and heat shock protein 60. By producing LAP in *L. paracasei*, infection of *Listeria* was decreased by about 44 to 46%. To reduce the infection of ETEC bacteria, *L. acidophilus* was engineered to produce the K99 fimbriae from ETEC. The K99 fimbriae from *L. acidophilus* reduced the attachment of ETEC bacteria to porcine intestine (Chu et al. 2005). Similarly, recombinant *L. casei* producing the ETEC adhesins K99 and K88 effectively reduced ETEC infection in mouse model (Wen et al. 2012).

By producing a toxin receptor or toxin binding site in probiotics, this can directly neutralize pathogens or enhance the host defense system leading to reduced the infection of pathogens. A recombinant non-pathogenic *E. coli* reduced the infection of shigatoxigenic *E. coli* by expressing galactosyl transferase genes from *Neisseria gonorrhoeae* which encode for a toxin-specific receptor (Paton et al. 2000). Moreover, one group expressed a cholera toxin receptor in probiotic *E. coli* and reduced the infection of virulent *V. cholera* by binding to cholera (Focareta et al. 2006). Similarly, lipopolysaccharides that are capable of binding the enterotoxin of ETEC were engineered to be produced in nonpathogenic *E. coli* and could neutralize the enterotoxin up to 93% (Paton et al. 2005). Moreover,

producing a toxin fragment in probiotics increased the IgA levels of the host and led to an enhanced defense system to protect the host (Norton et al. 1995).

(3) Improvement of stress tolerance

Improving the survivability of probiotics can also enhance the activity of probiotics in the host intestine. Therefore, some studies have tried to improve the stress tolerance of probiotics toward temperature, oxygen, or acidic conditions. Desmond et al. selected genes that are related to heat stress to impact heat tolerance and solvent resistance (Desmond et al. 2004). *L. paracasei* NFBC338 was engineered to produce heat shock protein chaperones (GroES and GroEL), and the recombinant probiotic survived almost 10 to 54 fold longer than the natural *L. paracasei* NFBC338. Recombinantly expressing osmolarity related genes could enhance the survivability of probiotics various stress conditions. *L. salivarius* UCC118 was engineered to express the listerial betaine uptake system, which is linked to the salt tolerance of *Listeria* (Sheehan et al. 2006). The recombinant probiotic showed resistance to several stress conditions such as osmotic and freezing conditions. Similarly, expressing the trehalose synthesis gene of *E. coli* in *L. lactis* enabled it to survive better in bile, acidic, cold and heat conditions (Carvalho et al. 2011; Termont et al. 2006).

(4) Immunomodulation

In the digestive tract, IBD is caused by inflammation which comes from chronic inflammatory disorders. IBDs such as Crohn's disease and ulcerative colitis occur when the inflammatory response is not controled (Mathipa and Thantsha 2017). Therefore, providing the continuous production of immunogenic molecules is needed to modulate humoral and cellular immune responses before infection. Several studies have reported on this concept to overcome digestive diseases. One group produced elafin in a recombinant *L. lactis* strain. Elafin is a protease inhibitor that is expressed in the epithelium of the intestines (Bermudez-Humaran et al. 2015). It is known to reduce inflammation in the intestine. Therefore, this group treated colitis mice with this recombinant *L. lactis* strain. The recombinant probiotic decreased the inflammatory parameters while anti-inflammatory cytokines such as IL-10 and TGF-β1 were increased compared to the wildtype probiotic strain. Another study induced IL-10 through recombinant engineering of *L. lactis* (Kumar et al. 2016). IL-10 is a cytokine that is expressed in many inflammatory responses and has an immunomodulatory role in the anti-inflammatory response in the host. Therefore, the *L. lactis* strain producing IL-10 decreased the clinical symptoms of colitis in mice. However, the biological processing of probiotics is still much too complicated, and there are a rising concern regarding genetically modified products among many consumers.

Table 3. Biological application to enhance the activity of probiotics

Biological process	Probiotics	Application	Action	References
Production of antimicrobial compounds	*Saccharomyces cerevisiae*	*pedA*	Inhibit *L. monocytogenes* B73	Schoeman et al. 1999
	L. lactis	A3APO and alyteserin	Inhibit *E. coli* and *Salmonella*	Volzing et al. 2013
	L. casei	lactoferrin	Inhibit enteric pathogens	Chen et al. 2010
	E. coli	fused thioredoxin gene to *pedA*	Similar antimicrobial activity against native pediocin	Beaulieu et al. 2007
	L. lactis IL1403	fused mature pediocin with leader of lactococcin A	-	Horn et al. 1999
	E. coli	LasR gene	Inhibit *Psedomonas aeruginosa*	Goh et al. 2012
	E. coli	Ganglioside	Protect host against diarrheal infection	Amalaradjou and Bhunia 2013
	L. reuteri	Heat-stable (ST) and heat-labile (LT) enterotoxins	Inhibit enterotoxicity bacteria	Amalaradjou and Bhunia 2013
Preventing colonization and toxin neutralization	*L. lactis*	Internalin A	Inhibit *L. monocytogenes*	Innocentin et al. 2009
	L. lactis	Surface associated flagellin	*Inhibit E. coli* and *S.enterica*	Sanchez et al. 2011
	L. paracasei	*Listeria* adhesion protein (LAP)	Inhibit *Listeria*	Koo et al. 2012
	L. acidophilus	K99 fimbriae	Inhibit ETEC bacteria	Wen et al. 2012 Chu et al. 2005
	L. casei	ETEC adhesins K99 and K8	Inhibit ETEC bacteria	Wen et al. 2012

	non-pathogenic *E. coli*	galactosyl transferase genes	Inhibit shigatoxigenic *E. coli*	Paton et al. 2000
	E. coli Nissle 1917	Receptor GM1	Inhibit *V.cholerae*	Focareta et al. 2006
	nonpathogenic *E. coli*	lipopolysaccharides	neutralize the enterotoxin	Paton et al. 2005
Improve stress tolerance	*L. paracasei* NFBC338	GroES and GroEL	survive almost 10 to 54 fold higher	Desmond et al. 2004
	L. salivarius UCC118	listerial betaine uptake system	Survive higher in stress conditions	Sheehan et al. 2006
	L. lactis	trehalose synthesis gene	Survive higher in stress conditions	Termont et al. 2006 Carvalho et al. 2011
Immunomodulation	*L. lactis*	Elafin	reduction in inflammation	Bermudez-Humaran et al. 2015
	L. lactis	IL-10	Prevent colitis	Kumar et al. 2016
	L. lactis	TGF-β	reduction in inflammation and colitis	Bermudez-Humaran et al. 2015
	L. lactis	Anti-TNF-α nanobodies	Reduced the colonic inflammation	Vandenbroucke et al. 2010

This table is modified from Mathipa and Thantsha (2017)

2) Physical methods

Optimizing the physical growth conditions of each bacterium is essential to enable the probiotics to achieve the best quality. Among physical methods, the optimization of various parameters such as pH, temperature, aeration, and incubation period can influence the activity of the probiotics including the production of bacteriocin. Already, there are many studies optimizing the growth conditions of each bacterium. For a higher production of pediocin, the bacteriocin from *Pediococcus* spp., the aeration condition, temperature and batch condition could change the amount of pediocin. Anaerobic and fully aerobic conditions were unfavorable for pediocin production, while semi-aerobic conditions led to the highest concentration of pediocin (Anastasiadou et al. 2008b). However, probiotics that produce nisin or amylovorin are more favorable to produce bacteriocins in oxygen enriched medium conditions although they are also lactic acid bacteria (Amiali et al. 1998; DeVuyst et al. 1996). Temperature is another changeable condition to increase the activity of probiotics. Among the same species, different strains can have a different favorable temperature (Anastasiadou et al. 2008b; Papagianni and Anastasiadou 2009). *Pediococcus acidilactici* NRRL B562, *Pediococcus acidilactici* H, *Pediococcus pentosaceus* ST18, and *Pediococcus damnosus* NCFB1832 produced the highest level of pediocin at 30 ℃ while the highest level for *Pediococcus pentosaceus* Pep1 was achieved at 35 ℃ and for *Pediococcus acidilactici* F and *Pediococcus pentasaceus* at 37 ℃. Moreover, for Pediococcus acidilactici, the concentration of pediocin SA-1 was

different based on the incubation period (Anastasiadou S et al. 2008). The amount of pediocin SA-1 was highest at 14 h and was stable until 28 h but decreased from this time point forward. Not only *Pediococcus* spp., but also other bacteria species such as *Lactobacillus* spp. and *Bacillus* spp. are strongly influenced by temperature, pH and incubation period (Arokiyamary and Sivakumaar 2011; Cladera et al. 2004; Krier et al. 1998). In contrast, a high hydrostatic pressure treatment in *Enterococcus* strains(Rubio et al. 2013) and *Weissella viridescens* (Stratakos et al. 2016) was shown to enhance the production of bacteriocin with an antimicrobial effect against *Listeria monocytogenes*.

3) Chemical method

Optimizing fermentation conditions with different chemical supplements in growth media can be another way to enhance the activity of probiotics (Table 4). Anastasiadou et al. studied the influence of various nutrient supplements on the production of pediocin by *Pediococcus acidilacitici* (Anastasiadou et al. 2008a). Among the various carbohydrates of glucose, sucrose, fructose, galactose, and glycerol, using a glucose carbon source produced highest pediocin concentration. Moreover, this group also found out that the different types of salts used in the media influence the production of pediocin. The addition of $MnSO_4H_2O$ resulted in a significant increase in the pediocin amount compared to the other supplements. Moreover, culture pH is an important parameter for probiotics to exhibit their best activity. Different pHs can change the enzyme activity of probiotics (Guerra and Pastrana 2003; Nelson and Lorenzo 2002). Therefore, finding a medium that can have a reduced pH that is adequate for each bacterium can also be important. Although several carbohydrates have been adopted as part of a chemical method to enhance the health benefits of probiotics, a detailed analysis of the effects of carbohydrate treatment on probiotics has not yet been done. Moreover, there have been no investigations on the alterations in metabolite production in probiotics using carbohydrate nanoparticles.

Besides enhancing the growth or activity of probiotics by biological, physical, and chemical compounds supplementation, protecting probiotics to survive in acidic gastric conditions with enzymes and bile acid is another important issue. In

general, to enhance the survivability of probiotics, many studies have used chemical materials to form capsules, tablets or gels (Neerja et al. 2012). There are two main ways to protect probiotics, one such method is entrapping probiotics within materials (Amalaradjou and Bhunia 2012). Another way is to encapsulate the probiotics by coating them. Encapsulation is a more common method to protect probiotics because it is easy to stabilize and control them once encapsulated. The materials that are used to entrap or encapsulate probiotics are mostly alginate, carrageenan, gelatin, chitosan, whey proteins, cellulose acetate phthalate, and starches (Amalaradjou and Bhunia 2012). To protect probiotics with alginate, is is used as a gel type or with a chitosan coating. Forming alginate into a gel enabled it to protect probiotics from gastric conditions (Gbassi et al. 2009). In addition, by capsulating probiotics with alginate and chitosan, it was possible to form pH-sensitive a microcapsule which protects probiotics from the gastric condition and releases them at intestinal pH (Cui et al. 2018). By making an alginate microcapsule, chitosan stabilizes alginate at a pH above 3; therefore, a more protective microcapsule against acidic pH can be made. Cellulose acetate phthalate (CAP) is a very common coating material used in many industries such as pharmaceutical drugs and vaccines (Neurath et al. 2001). Because CAP has phthalate on its structure, the carboxylic group contains pH sensitive characteristics. Therefore, encapsulating probiotics with CAP could enhance the survivability of the probiotics in gastric conditions and easily release the probiotics in intestinal conditions. By using materials that are pH sensitive and mucoadhesive, they could have enhanced adhesion in intestinal conditions.

Coating probiotics with hypromellose phthalate has shown pH-sensitive and mucoadhesive properties because hypromellose has good mucoadhesive properties (Alli 2011). Moreover, using materials that contain thiol groups like mercaptosuccinic acid or adding a thiol group to the outside of the coating materials is also effective for enhancing adhesion (Mathieu et al. 2018). The mucus layer in the intestine has thiomer in the mucin which can bond as a disulfide bond with another thiol group. This bonding can enhance probiotics to stay longer in the intestinal tract.

Using tablets is one of the most famous strategies for entrapping probiotics. A tablet made of carboxylate methyl high amylose starch and chitosan efficiently delivered probiotics to the intestine (Calinescu and Mateescu 2008). Additionally, a tablet made of hydroxypropyl methylcellulose phthalate 55 (HPMCP 55) also protected probiotics from harmful gastric conditions and released them in the intestinal conditions *in vitro* and *in vivo* (Jiang et al. 2017). Tableting enhanced the colonization of the probiotics in the digestive tract compared to probiotics taken by themselves. Moreover, tableting provided a longer stability to the probiotics, because they had a better viability after 6 months compared to the probiotics taken by themselves. Hence, protecting probiotics from acidic conditions could also enhance the viability of the probiotics providing a longer storage time (Jonganurakkun et al. 2006).

Table 4. Chemical application to enhance activity and viability of probiotics

Chemical process	Agents	Probiotics	Action	References
Nutrient supplement	Carbon source	*Pediococcus acidilacitici*	Produced higher bacteriocin concentration	Anastasiadou et al. 2008a
	Salts	*Lactococcus lactis* and *Pediococcus acidilactici*	Optimized adequate pH drop with salts for probiotics to produce higher bacteriocins	Guerra and Pastrana 2003
		Lactococcuslactis subsp. *lactis* CECT 539 and *Pediococcus acidilactici* NRRL B-5627		Guerra and Pastrana 2003; Nelson and Lorenzo 2002
Encapsulation	Calcium alginate beads	*Lactobacillus plantarum* strains	Protect probiotics from harsh condition	Gbassi et al. 2009
	Alginate chitosan	*Pediococcus acidilactici*	Higher survival and improved the production of bacteriocins	Cui et al. 2018
	Cellulose acetate phthalate (CAP)	*Bifidobacterium*	Protect probiotics from harsh condition	Rao et al. 1989
	Hypromellose phthalate	*Lactobacillus rhamnosus*	pH-sensitive and mucoadhesive	Alli 2011
Entrapping (Tablet)	Carboxymethyl high amylose starch and chitosan	*Escherichia coli*	Efficiently deliver probiotics to the intestine	Calinescu and Mateescu 2008
	Hydroxypropyl methylcellulose phthalate 55 (HPMCP 55)	*Pediococcus acidilactici* (GS1)	pH-sensitive to protect probiotics and provided good storage viability	Jiang et al. 2017

4. Prebiotics

1) Definition of prebiotics

Prebiotics are generally defined as non-digestible materials that stimulate the growth or activity of probiotics or other microorganisms in the gastrointestinal (GI) tract to confer favorable health effects on the host (Gibson and Roberfroid 1995). Gibson et al. defined prebiotics as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon” at the beginning. Nowadays, the range of prebiotics is broader than before and are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al. 2017). However, there still are conflicting interests in terms of the concept and range of prebiotics, and the definition of prebiotics seems to be expanding over time.

Generally, prebiotics can be classified into carbohydrate compounds and non-carbohydrate ones (Louis et al. 2016) (Table 5). The carbohydrate compounds contain inulin-type fructans, galacto-oligosaccharides, glucose-derived oligosaccharides, starch, pectic oligosaccharides, oligochitosan, and pullulan. The non-carbohydrate compounds contain fatty acids (CLA and PUFA), polyphenols, phytochemicals, and flavonoids although the effect of prebiotics does not come from the fermentation of these compounds (Jang et al. 2016).

The function of prebiotics is mainly to promote the growth or activity of probiotics. Prebiotics alters the gut environment by generating SCFAs or changing the pH, and viscosity of the gut to interact with other food components. Basically, the health benefits are largely mediated by SCFAs produced by gut microbiota through the metabolism of prebiotics (Gourbeyre et al. 2011). In general, dietary fibers consisting of carbohydrates are widely used as prebiotics because the fermentation of carbohydrates generates SCFAs. Interestingly, SCFAs such as acetate or propionate, are selectively produced by LAB of the *Lactobacillus* and *Bifidobacterium* genera, while butyrate is produced by the *Clostridium* and *Eubacterium* genera. Moreover, prebiotics alter the gut environment, such as the pH, viscosity, gut transit and interactions with other food components (Gourbeyre et al. 2011).

Table 5. Type of prebiotics

Category	Compound	Composition	Source		Reference
Carbohydrate	Inulin	β(2→1) α(1→2)	Jerusalem artichoke, Chicory etc.	-	Ramnani et al. 2010
	Dextran	α(1→6) α(1→3) α(1→4)	Synthesized by *Leuconostoc mesenteroides* and *Streptococcus mutans*	-	Howarth et al. 2001
	Fructo-oligosaccharide (FOS)	β(2→1)	Bamboo shoots, Vegetables (Jerusalem artichoke, garlic, chicory), Milk	Substrate of Inulin degradation or trans-fructosylation	Sabater-Molina et al. 2009
	Galacto-oligosaccharide (GOS)	β(1→3) β(1→4) β(1→6)	Human and Bovine milk	Synthesized form β-D- Galactosidase	Elli et al. 2008
	Isomalto-oligosaccharide (IMO)	α(1→6)	Rice miso, Soy sauce	Hydrolysate of starch	Wu et al. 2017
	Xylo-oligosaccharide (XOS)	β(1→4)	Bamboo shoot	Hydrolysate of Xylan	Lecerf et al. 2012

	Chito-oligosaccharide	β(1→4)	-	Hydrolysate of Chitin	Thongsong et al. 2018
	Soy oligosaccharide	α(1→6)	-	Divide into stachyose and raffinose	Ma et al. 2017
Non-carbohydrate	CLA and PUFA	-	Sunflower seed oil, Beef, Milk	Omega-3,6,9	Gibson et al. 2017
	Polyphenolic and phytochemicals	-	Fruits (berries, oranges, lemons), Herbs	Curcumin, Resveratrol	Thilakarathna et al. 2018 Carrera-Quintanar et al. 2018
	Flavonoids	-	Onions, Wine, Chocolate, Fruits, Soy food	Cacao-drived flavonols	Tzounis et al. 2011

2) Inulin

Inulin is a group of naturally occurring polysaccharides produced by many types of plants such as chicory root, Jerusalem artichokes, leeks, and garlic. Inulin is a combination of fructose with an α-1,2 bond and sucrose in the form of glucose and fructose in the non-reducing terminal and is connected to the β-2,1 bonds of fructans. They are known as prebiotics because they promote the growth of beneficial intestinal bacteria, and increase calcium absorption and possibly magnesium absorption (Slavin 2013) (Table 6). Moreover, inulin is also used as synbiotics with many probiotics. Synbiotic is a combination of probiotics and prebiotics to have a synergistic effect on promoting the activity of probiotics and increasing the production of SCFA (Markowiak and Slizewska 2017).

Table 6. Inulin used as prebiotics

Material	Subject	Result	Reference
DP 2 and 60(inulin), 2 and 20(oligofructose)	Human	Increase *Bifidobcteria* and benefical genus	Gibson 1999
DP 4, 8, 16, 23	Rat	Improve *Bifidobacteria* in DP 8, 16, 23 DP4, 8, 16 significantly increased immunoglobulin production	Ito et al. 2011
Inulin extract from chicory	Pig(growing pigs 30-70kg)	3%, higher daily weight gain, lower cholesterol, highest level of omega-3/omega-6	Grela et al. 2013
Raftilose synergy 1 (oligofructose-enriched inulin) and probiotics	Rat	Reduce colon cancer and decrease carcinogenesis	Femia et al. 2002
FOS, inulin, and probiotics	Rat	Cecal *Bifidobacterium* or *Lactobacillus rhamnosus* were increased and enhanced colonic β-defensin1 and muc4 gene expression	Paturi et al. 2015

3) Dextran

Dextran is a complexly branched glucan (polysaccharide made of many glucose molecules). It is comprised of chains in various lengths from 3 to 10,000 KDa. The straight chain consists of α-1,6 glycosidic linkages between the glucose molecules, while branches begin from the α-1,3 and α-1,4 linkages. Dextran was first discovered by Louis Pasteur as a microbial product of wine, and now, it is synthesized from sucrose by certain lactic acid bacteria such as *Leuconostoc mesenteroides* and *Streptococcus* spp. These linkages cannot be digested by pancreatic enzymes in the upper gastrointestinal tract but can be fermented by gut microbiota. Dextran increased the number of *Bifidobacteria* (Sarbini et al. 2014). Moreover, they were able to alter the composition of microbiota in obese humans by increasing the numbers of *Bacteroides–Prevotella* and decreasing those of *Faecalibacterium prausnitzii* and *Ruminococcus bromii/R. flavefaciens* (Sarbini et al. 2014). Moreover, a dextran supplement increases the SCFA concentration which is a role of prebiotics. Therefore, dextran is considered as prebiotics (Olano-Martin et al. 2000; Sarbini et al. 2014).

5. Characteristics of nanoparticles

1) Nanoparticles

Besides enhancing the probiotics activity through biological, physical, and chemical methods, there has been growing interest in using antimicrobial nanoparticles to inhibit pathogenic infection.

Generally, nanoparticles have been used for biomedical applications because of their interesting characteristics, which enable them to overcome various biological barriers. Formulating linear polymers into nanoparticles are already used in many applications such as drug and gene delivery systems because they have many advantages compared to linear polymers (Zhu et al. 2017). Besides just using nanoparticles as a delivery carrier, nanoparticles can enhance their own activity. The structure of nanoparticles enables cells to recognize them better, and their binding or internalization capabilities also increase (Ren et al. 2016). Therefore, nanoparticles enhance the bioactivity of compounds. Furthermore, nanoparticles can provide a new avenue for synergistic therapies such as photodynamic therapy or for diagnostic purposes (Lam et al. 2018).

Nanoparticles can be synthesized in two different ways. First, a top-down method is using nanofabrication tools to control large dimension particles to produce nanosized particles (Biswas et al. 2012). A top-down method can be controlled by external methods such as pressure, optical, electron beam, soft, nanoimprinting, scanning probe, and block copolymer lithography to create

nanoscale structures with the desired shapes and characteristics. The top-down approach can be done a large-scale production with multi-directional pattering and nanoparticles having an equal size (Biswas et al. 2012). On the other hand, a bottom-up method synthesizes and assembles molecular compounds into more complex nanoscale particles (Biswas et al. 2012). Bottom-up nanofabrication techniques such as self-assembly, atomic layer deposition, sol–gel nanofabrication, and DNA-scaffolding are commonly used (Biswas et al. 2012).

For these reason, to enhance the antimicrobial activity of polymers, developing nanoparticles using nanofabrication techniques may induce multivalent interactions in multiple ligands or receptors. Additionally, nanoparticles may result stronger electrostatic interactions with anionic microbial membranes.

2) Mechanism of antimicrobial nanoparticles

Antimicrobial nanoparticles may be effective in replacing antibiotics for the treatment of many infectious diseases. Antimicrobial nanoparticles destroy bacteria by inducing toxicity through various mechanisms such as interacting with the cell barrier, penetrating or internalizing into cells, regulating biosynthesis, and inhibiting the formation of biofilms (Figure 9).

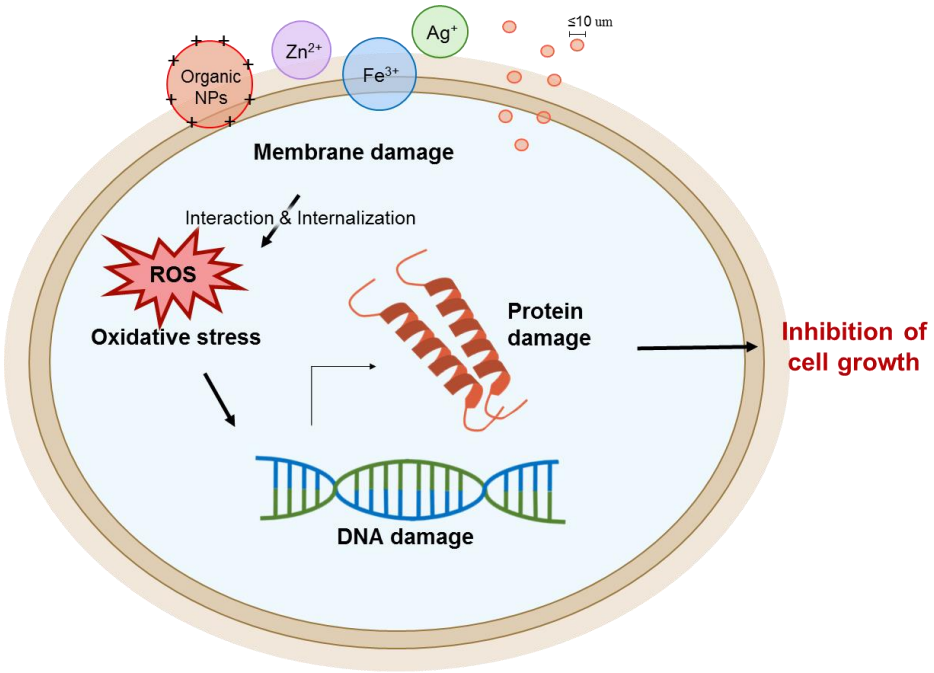


Figure 9. Antimicrobial actions of nanoparticles. Cationic nanoparticles can easily interact and are internalized by the bacterial cell membrane. After the interaction, nanoparticles disrupt cell metabolism by increasing oxidative stress. Additionally, the DNA and protein damage from the nanoparticles inhibits cell growth. ROS: Reactive oxygen species

(1) Interaction with the cell barrier

The bacteria cell wall and membrane provides structural support such as strength, rigidity, and shape and protects against osmotic stress or mechanical damage (Hajipour et al. 2012). They also can work as a filtering mechanism. According to the characteristics of the cell wall thickness, bacteria can be divided into two main categories, Gram-positive and Gram-negative bacteria (Figure 10). The outer surface of both bacteria is charged negatively. The cell wall of Gram-positive bacteria contains a thick layer of peptidoglycan which contains two types of teichoic acids, lipoteichoic acid and wall associated teichoic acid. Due to the phosphate in teichoic acids the Gram-postivie cell wall has a negative charge. For Gram-negative bacteria, the outer membrane of the bacteria contains lipopolysaccharides which provide a negative charge. Many metal ion nanoparticles have a positive charge on their surface; therefore, they can easily interact with the cell membrane by electrostatic attraction and disrupt cell metabolism. Hence, interacting with the cell wall can be one of the targeting methods to destroy bacteria with nanoparticles.

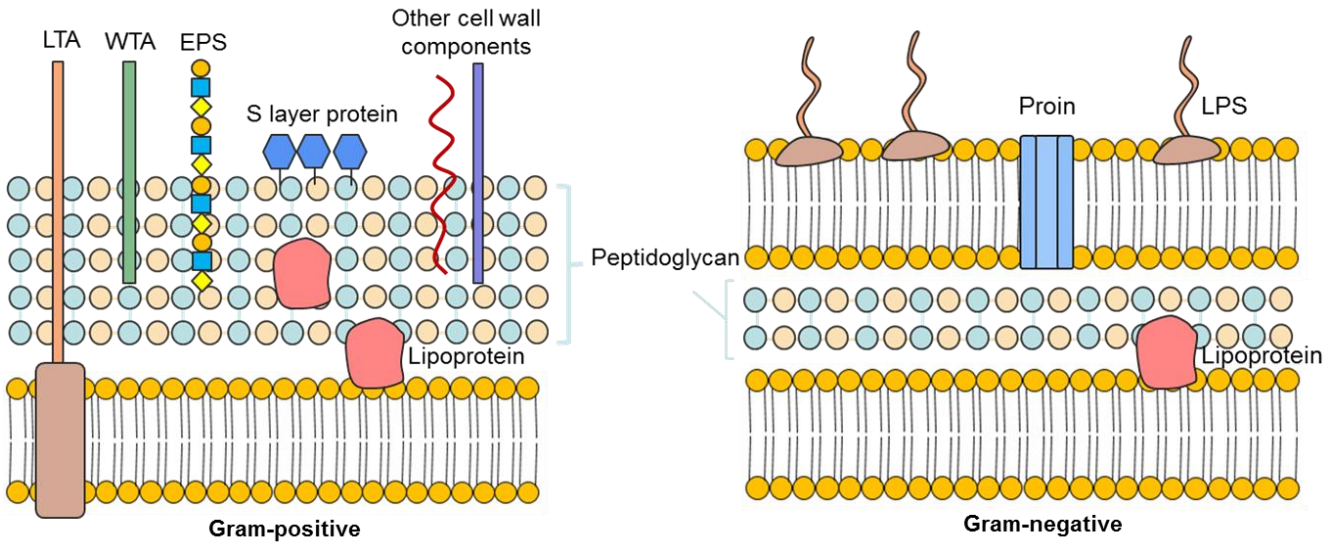


Figure 10. Bacterial cell wall components. Cell wall components of Gram-positive and Gram-negative bacteria varies from the cell wall thickness and components. They consist various sugars, proteins, lipids, etc in common, while Gram-positive bacteria contains teichoic acid in the cell wall. Gram-negative bacteria consists LPS in their outer membrane. LTA, Lipoteichoic acid; WTA, Wall teichoic acid, EPS, exopolysaccharide, LPS, Lipopolysaccharide

(2) Internalization of nanoparticles

Nanoparticles can be internalized into the cell by diffusion or adsorption after the interaction with the cell wall. Small sized nanoparticles less than 10 nm can easily diffuse into the cell without being affected by the barrier. Silver nanoparticles (Ag NPs) less than 10 nm could pass through the pores in the cell membrane and generate reactive oxygen species (ROS). Another mechanism is by absorption, in which a specific receptor or functional group in the cell recognizes the nanoparticles and internalizes them into the cell. Zinc ions have a high affinity with the thiol group of proteins; therefore, zinc ions or silver ion nanoparticles are able to attach or internalize into the cell then cause cell disruption such as destroying their inherent function which eventually leads to cell death (Padmavathy and Vijayaraghavan 2011). Moreover, silver ion nanoparticles can be internalized into cells through the reaction of Coulomb gravity, which changes the structure of proteins or enzymes (Jung et al. 2008).

(3) Inhibit and regulate the synthesis of genes

After the interaction and entering into the cells, the synthesis of genes and proteins are affected. When silver ions or nanoparticles enter the cells, a subunit of the ribosome and other enzymes are affected (Shrivastava et al. 2007). The interaction with ribosomal proteins and other proteins may induce cell disorder such as collapsing the membrane potential or reducing the ATP level by increasing the ATPase activity. Besides the interaction with proteins, nanoparticles may

mutate or regulate genes after entering to cells. The presence of TiO_2 nanoparticles mutated several areas of the bacterial genome which led to the modification of RNA and protein (Iram et al. 2015). Iron or silver nanoparticles are known to regulate gene expression by interacting with antioxidant genes, metal transporters, metal reduction enzymes, and the ATPase pump and upregulates oxidative stress to stimulate superoxide (Leuba et al. 2013; Nagy et al. 2011).

(4) Inhibit formation of biofilms

Bacteria form biofilm structures to have resistance against foreign chemicals. The biofilms of most bacteria are negatively charged in the matrix surface of the biofilms. Few bacteria such as *Staphylococcus epidermidis* may have a positive charged biofilm surface. Therefore, some metal ion nanoparticles can interact with negatively charged biofilm surfaces (Kumar et al. 2017). However, a biofilm can be resistant to a small amount of nanoparticles. Among the various nanoparticles, superparamagnetic iron oxide nanoparticles coated with silver or gold ions have shown the highest antimicrobial activity against biofilms (Park et al. 2011).

3) Antimicrobial nanoparticles

Antimicrobial nanoparticles can have antimicrobial activity through those interactions and affect bacterial cells. Both organic and inorganic nanoparticles are known to have antimicrobial activity against bacterial cells. However, the zeta potential, shape, size, and roughness can make a difference in the intensity of the interaction or in the subsequent effect leading to a difference in antimicrobial activity (Wang et al. 2017).

(1) Organic nanoparticles

Before, conjugating conventional antibiotics with synthetic polymers was used to overcome the resistance of antibiotics. However, for organic nanoparticles, a recent strategy is to mimic the chemical structure of antimicrobial peptides (Table 7).

For organic antimicrobial nanoparticles, the self-assembly method is largely used. Because organic polymers have less toxicity than that of metal ions and are biodegradable, introducing cationic polymers or functional groups to polymers are used to target bacteria. Cationic amphiphilic polycarbonate without metal was synthesized by Nederberg et al. A poly(5-methyl-5-(3-chloropropyl) oxycarbonyl-1,3-dioxan-2-one) block was sandwiched between two poly(trimethylene carbonate) (PMTC) blocks (Nederberg et al. 2011). Due to the cationic residue, they were able to interact electrostatically at the bacterial cell membrane. The cell wall and membrane disruption led to cell death including *Bacillus subtillis*,

Enterococcus faecalis, *S. aureus*, and MRSA. Moreover, to enhance the zeta potential in an aqueous solution, a cholesteryl group was introduced to cationic polycarbonate oligomers (Coady et al. 2014). The sizes of the synthesized nanoparticles were about 10 to 11 nm. The nanoparticles exhibited a higher antimicrobial activity than that of the polycarbonate polymers against *S. aureus*, *E. coli*, and *P. aeruginosa*. This group also demonstrated that the hydrophobic and hydrophilic balance is important for enhancing the antimicrobial activity. Oligomers with a degree of polymerization (DP) above 10 showed a higher antimicrobial activity. However, it should be noted that already several polymers have been found to have antimicrobial activity as individual chains. Polycarbonate based micelles, poly(ethylene oxide)-*b*-poly(ε-caprolactone)-*b*-poly[(2-*tert*-butylaminoehtyl) methacrylate] (PEO-*b*-PCL-*b*-PTBAM), has a biodegradable and antimicrobial characteristic (Yuan et al. 2012). Due to the cationic secondary amine group in the PTBAM block, they interact with the bacterial cell membrane and have an antimicrobial activity against *E. coli* and *S. aureus*. PEG is a well-known compound that is used in many applications because it has a low toxicity against mammalian cells. Nanoparticles were made using PEG and functionalizing with amphiphilic polypeptides such as lysine and phenylalanine (Costanza et al. 2014). The size of the micelle was about 50 to 200 nm. The nanoparticles were more active against Gram-positive strains, and the mechanism was through membrane disruption. Chitosan and chitosan derivatives are another polymer that is used to confer antimicrobial activity and biocompatibility. However, chitosan has poor solubility in low pH conditions, and thus, conjugating

chitosan with other compounds is used (Lam et al. 2018). Chitosan was functionalized with poly(methacryloyloxy ethyl trimethylammonium chloride) (PMDC) (Lin et al. 2015). The micelles size was 120-200 nm in acetone. The nanoparticles inhibited the growth of *E. coli* compared to chitosan alone. Increasing the positive charge density through the addition of ammonium groups on PDMC may improve the interaction with the cell membrane.

Furthermore, most self-assembled nanoparticles have a hydrophilic and hydrophobic part to them, enhancing the activity of nanoparticles encapsulation, or conjugating drugs to nanoparticles has also been used. Polypeptide-grafted chitosan-based nanocapsules were made by (Zhou et al. 2013). The nanocapsules had antimicrobial activity against *E. coli* and *S. aureus* compared to a liner polypeptide. The positive charge was more induced due to the chitosan backbone, therefore, could enhance the antimicrobial activity. Additionally, the nanocapsules could entrap hydrophilic moieties and anticancer, and anti-epileptic drugs inside the nanocapsules.

Table 7. Organic antimicrobial nanoparticles

Nanoparticles	Physicochemical properties of NPs	Antimicrobial activity	Applied dosage	Reference
PMTC and PMTC block	43-402 nm	*Bacillus subtilis*, *Enterococcus faecails*, *S.aureus*, MRSA, *Cryptococcus neoformans*	4.3 – 10.8 µM	Nederberg et al. 2011
Cholesteryl cationic polycarbonate oligomers	10-11 nm	*S. aureus*, *E. coli*, *P. aeruginosa*, *Candida albicans*	3.9 – 250 µg/mL	Coady et al. 2014
PEO-*b*-PCL-*b*-PTBAM copolymer	-	*E. coli*, *S. aureus*	-	Yuan et al. 2012
PEG-poly(amino acid) copolymers (50~60% hydrophobic content)	50-200 nm	*S. epidermis*, *B. subtilis*, *Klebsiella pneumonia*, MDR *P. aeruginosa*	5 – 92 µM	Costanza et al. 2014
PPEG/PQA4c, PQA8c, PQA12c	-	*E. coli*, *S. aureus*	39.1, 19.5µg/mL	Wan et al. 2015
PDMC-functionalized chitosan	120-200 nm	*E. coli*		Lin et al. 2015
Polyion complex (PIC) micelles		*E. coli*	19.7 µg/mL	Li et al. 2014b
Terephthalamide-polylactide-polycarbonate triblock copolymers	Spherical 20.4 nm Rod 10 nm	*S. aureus*, MRSA, VRE, *E. coli*, *C. neoformans*	Spherical (20-150µg/mL) Rod (20-100µg/mL)	Fukushima et al. 2012

$PMEO_2MA$ and PTBAM copolymers	240 nm	*E. coli*, *S. aureus*	0.25 mg/mL	Wang et al. 2016
PEO_{43}-*b*-$PDEA_{20}$-*b*-$PTBAM_{30}$-based polymersome	30-40 nm	*E. coli*, *S. aureus*	0.15, 0.6 mM	Zhang et al. 2013
PLL-*stat*-PPhe grafted chitosan-based nanocapsules	-	*E. coli*, *S. aureus*	16 μg/mL	Zhou et al. 2013
$TPE_{core}P(qDMAEMA\text{-}co\text{-}BMA\text{-}co\text{-}Gd)_{arm}$ star polymers	-	*E. coil*, *P. aeruginosa*, *S. aureus*	5.5, 0.12, 30 μg/mL	Li et al. 2014a

(2) Inorganic nanoparticles

Many types of research have already revealed that inorganic nanoparticles such as metal nanoparticles (Ag, ZnO, TiO_2, and CuO) themselves have antimicrobial properties against bacteria (Hajipour et al. 2012) (Table 8). These studies have revealed that the bactericidal ability was derived from the ionic interaction with the bacterial membrane(Sanyasi et al. 2016) or bacterial growth was abrogated by disrupting the membrane permeability (Palza 2015). They interact or penetrate into the bacterial cell membrane and induces ROS eventually leading to cell death.

However, metal nanoparticles have several limitations, such as aggregation and low stability *in vivo*. Recently, to expand their application coverage, metal or metal oxide nanoparticles have been coated or conjugated with polymers or bacterial proteins to prevent aggregation, promote stabilization, and reduce toxicity (Lam et al. 2018). These methods still can cause serious side effects in the host (Roy et al. 2003). Another limitation is that metal nanoparticles inhibit both pathogens and beneficial microbes (Travan et al. 2009). Hence, a balance between antimicrobial activity and biocompatibility is important. Therefore, alternative strategies are needed.

Table 8. Inorganic antimicrobial nanoparticles

Nanoparticles	Physicochemical properties of NPs	Antimicrobial activity	Applied dosage	Reference
ZnO (Zinc oxide)	< 100 nm	Halophilic bacterium spp. EM84	2-5 mM	Sinha et al. 2011
		B. subtilis	10 mM	
	10–20 nm	*P. aeruginosa*	1-4.25 mM	Feris et al. 2010
	~20 nm	*E. coli*	20 mg/L	Jiang et al. 2009
	25-40 nm	*S. typhimarium*	8 and 80 ng/mL	Kumar et al. 2011
	50-70 nm	*P. putida* KT2442	10 mg/L	Gajjar et al. 2009
Ag (Silver)	< 100 nm	Halophilic bacterium spp. EM84	2-5 mM	Sinha et al. 2011
		B. subtilis	10 mM	
	2-4 nm	*B. subtilis*	-	Ruparelia et al. 2008
	43 nm	*K. pneumoniae*	30 mg/L	Khan et al. 2011
	1-10 nm	*P. aerguginosa*	25-100 mg/L	Morones et al. 2005
		E. coli	25-100 mg/L	
	5-100 nm	Waste water biofilm bacteria	1-200 mg/L	Sheng and Liu 2011
	~10 nm	*P. putida* KT2442	1 mg/L	Gajjar et al. 2009
TiO (Titanium oxide)	40-60 nm	*B. subtilis*	-	Khan et al. 2011
	~20 nm	*M. smegmatis*	20 mg/L	Wu et al. 2010
	10-25 nm	*P. aeruginosa*	10 mg/L	Tsuang et al. 2008
	~50 nm	*E. coli*	20 mg/L	Jiang et al. 2009

	20 nm	*E. coli*	10mg/L	Tsuang et al. 2008
	40-60 nm	*S. typhmurium*	8 and 80 ng/mL	Kumar et al. 2011
	< 25 nm	*C. metallidurans* CH34	8 mg/mL	Simon-Deckers et al. 2009
Si (Silica)	80-100 nm	*S. epidermidis*	-	Hetrick et al. 2009
	17-69 nm	*E. coli*	< 2 mg/mL	Song et al. 2009
		S. aureus	< 2 mg/mL	Song et al. 2013
	15 nm	*E. coli*	0.75 mg/mL	Song et al. 2011
		S. aureus	1 mg/mL	
CuO (copper oxide)	8-10 nm	*B. subtilis*	-	Ruparelia et al. 2008
NiO (Nickel oxide)	~20-30 nm	*E. coli*	20 mg/mL	Wang et al. 2010

This table is modified from Hajipour et al. (2012)

Study 1. Investigation of the effect of inulin nanoparticles on probiotics activity

1. Introduction

Prebiotics are generally defined as non-digestible materials that stimulate the growth and/or activity of probiotics and/or other microorganisms in the gastrointestinal tract to confer favorable health effects on the host(Gibson and Roberfroid 1995). Prebiotics are known to alter the gut environment by generating SCFAs or changing pH, viscosity of gut to interact with other food components. Although a variety of compounds have been used as prebiotics, the most frequently used and studied dietary fiber is inulin. Inulin is naturally present as a polysaccharide in many plants, although the main industrial source is chicory root. Typically, inulin is a fructan derivative consisting of fructosyl residues (n=2–60) linked by β (2→1) bonds and a glucosyl residue as an end group. Due to the β (2→1) linkages in inulin, it is not digested by pancreatic enzymes in the upper GI tract. However, inulin can be fermented by the gut microbiota present in the colon and produces SCFAs, leading to stimulation the growth of specific bacterial populations in the host intestine and subsequently altering the host immune system.

One of the most challenging aspects of probiotics as a replacement for antibiotics is to enhance their antimicrobial activity against pathogens. Due to the improper use of antibiotics antibiotic-resistant bacteria was developed. Generally,

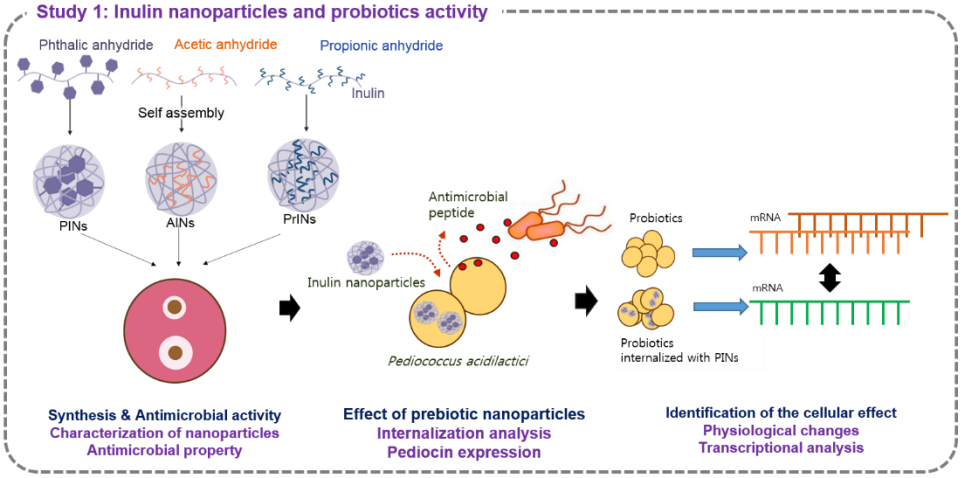
probiotics can directly affect the pathogens by producing antimicrobial substances such as bacteriocins. Moreover, they can modulate the host barrier function, immune systems, and to produce host antimicrobial peptides to defense the infection of pathogens (Oelschlaeger 2010). However, there are limitations on using probiotics as a replacement for antibiotics due to their low production of antimicrobial substances.

Polymeric nanoparticles have been widely used in biomedical applications because they can deliver chemotherapeutics, proteins, genes and contrast agents as cargoes to the desired place of action or in response to specific biological or external stimuli (Petros and DeSimone 2010). Particularly, polymeric nanoparticles can deliver hydrophobic drugs or macromolecules into the cells by overcoming the cellular barriers as they are internalized with cellular membranes by endocytosis (Elsabahy and Wooley 2012). Most importantly, polymeric nanoparticles can be easily formed by using the self-assembly method. They can be made by modifying hydrophilic polymers with hydrophobic groups, due to the hydrophobic interaction of hydrophobic groups in the inner cores of the polymeric nanoparticles. For instance, water-soluble hydrophilic polymer (inulin) can be incorporated with hydrophobic groups such as phthalates and form inulin nanoparticles through hydrophobic interactions of the phthalates.

The aim of the study 1 was to investigate the effect of inulin nanoparticles as prebiotics on probiotics property (Figure 11). In this study 1, four phthalyl inulin nanoparticles (PINs) were synthesized by their phthalyl contents. *Pediococcus*

acidilactici (PA), a probiotic that produces pediocin (antimicrobial peptide) was chose as probiotics. To analyze the changes in the antimicrobial activity of probiotics, PINs were treated to PA and was analyzed with various antimicrobial assays. Furthermore, the consequences of PINs on PA was explored at the genetic level by transcriptome analysis. Study 1 is the first report to demonstrate improved antimicrobial activity of probiotics using a prebiotic in nanoparticles form.

A



B

Study	Category	Item
Development and phenotypic analysis of prebiotic nanoparticles	Synthesis of inulin nanoparticles	Inulin, hydrophobic group (phthalyl anhydride, acetic anhydride, propionic anhydride)
	Characterization	HNMR, SEM, DLS, ELS
	Antimicrobial property	Co-culture assay (viable cell counts)
		Pathogen agar layered test (inhibition zone)
	Tracking internalization of PINs	Confocal microscope (FITC, curcumin)
		TEM analysis
		SEM analysis
Mechanism study of enhanced antimicrobial property	Effects of PINs on probiotics	Viable cell counts and pH
		SCFA (GC)
		Pediocin (protein, mRNA, activity)
		Hydrogen peroxide
	Transcriptional analysis	Scatter plot, volcano plot, RAST annotation
		HSP mRNA level

Figure 11. Overview of study 1. (A) Graphical abstract of the study 1. (B) Contents of study 1

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 inulin nanoparticles (PINs)

PINs were synthesized according to a previously described method(Sunny et al. 2016) with a slight modification (Figure 12). Briefly, inulin (1 g, MW=5000 $gmol^{-1}$) was added to 5 ml of dimethyl formamide, and then 0.2 ml of 5 % sodium acetate (w/v) was added as a catalyst for the reaction. Subsequently, phthalic anhydride was added to the inulin solution at various molar ratios, such as 0.3:1 (PIN1), 0.6:1 (PIN2), 1.2:1 (PIN3) and 2:1 (PIN4), to produce PINs with varying degrees of substitution of phthalate moieties in inulin. Four separate reactions were performed at 40 °C for 24 h under nitrogen. The produced PINs were dialyzed against distilled water at 4 °C for 24 h to form self-assembled nanoparticles of phthalic anhydride to inulin. Finally, the PINs were lyophilized and stored at -20 °C until use. Following the above protocol, acetyl inulin (AI)

and propyl inulin (PrI) were synthesized, and similarly, the synthesized molar ratio was 1:1 between acetate anhydride:inulin and propionic anhydride:inulin.

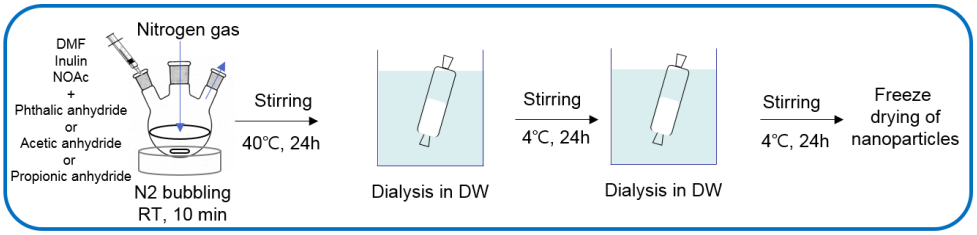


Figure 12. Synthesis procedure of inulin nanoparticles. Inulin nanoparticles was developed by conjugating three individual hydrophobic residues (phthalic anhydride, acetic anhydride, propionic anhydride) to inulin. By dialysis stage nanoparticles was form by self-assembly method. (DMF: Dimethylformamide, DW: Distilled water)

3) Characterization of PINs

The contents of the phthalyl group in PINs were confirmed by 600 MHz ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (AVANCE 600, Bruker, Germany). The surface topography of PINs was analyzed using a field-emission scanning electron microscope (FE-SEM) with SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). PINs were mounted on the stubs with adhesive copper tape and coated with platinum under a vacuum using a coating chamber (CT 1500 HF, Oxford Instruments, Oxfordshire, UK). 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). Following the above protocol, acetyl inulin (AI) and propyl inulin (PrI) were characterized.

4) Bacterial cultures

All bacterial strains were cultured in the corresponding medium: *Pediococcus acidilactici* 175 (PA, KCTC21088) in MRS broth, Gram-negative *Salmonella* Gallinarum in LB broth, and Gram-positive *L. monocytogenes* in BHI broth. All bacterial cultures were incubated at 37 °C in a shaking incubator (255 rpm) for 24 h prior to being applied to the subsequent experiments or stored at -70 °C in 15 % (v/v) glycerol.

5) Co-culture and agar diffusion test for antimicrobial activity

Antimicrobial activities of PA against *Salmonella* Gallinarum, ETEC K88 and *L. monocytogenes* strains were determined using the co-cultivation assay (Ditu et al. 2011) and agar diffusion test(Driscoll et al. 2012) with some modifications (Figure 13). To quantitatively compare the antimicrobial activity of PA against *Salmonella* Gallinarum by the co-cultivation assay, 2.0 x 10^5 CFU/ml of *Salmonella* Gallinarum was co-cultured with 2.0 x 10^5 CFU/ml of PA treated with or without 0.5 % (w/v) PINs or inulin in MRS broth for 8 h at 37 ℃ with aerobic condition in a shaking incubator (255 rpm). The degree of antimicrobial activity of PA against *Salmonella* Gallinarum in the co-culture could be directly measured by the survival rate of *Salmonella* Gallinarum. Hence, the co-culture samples were spread on MacConkey agar, incubated for 24 h at 37 °C and the number of *Salmonella* Gallinarum colonies was counted. For ETEC K88, same protocol was followed. To test the antimicrobial activity of PA against *L. monocytogenes* using the co-cultivation assay, *L. monocytogenes* and PA were cultured in BHI broth and exposed to similar conditions as described above. Finally, the co-culture samples were spread on Oxford agar, and the number of *L. monocytogenes* colonies was counted.

Alternatively, the agar diffusion test was used to determine whether PA cultures treated with or without PINs were able to inhibit the growth of pathogens on an agar plate *in vitro*. First, 120 µl of Gram-negative pathogen (2.0 x 10^8 CFU/ml) was spread on an LB agar plate. A paper disc was placed on the pathogen-spread

plate, and 120 μl (2.0 x 10^8 CFU/ml) of PA culture treated with or without 0.5 % (w/v) PINs or inulin was dropped onto the paper disc. After drying for 20 min at room temperature, the disc was cultured for 20 h at 37 °C. The zones of inhibition of pathogen growth, as a direct consequence of the antimicrobial activity of the PA cultures on the agar plate, were measured. Similarly, the same protocols were followed as above to observe the inhibitory effect on Gram-positive pathogen by PA cultures treated with or without 0.5 % (w/v) PINs or inulin, excluding the tests performed on BHI agar plates.

To examine the concentration-dependent antimicrobial activity of probiotics, PA (2.0 x 10^5 CFU/ml) were inoculated into 1 ml of MRS broth and treated with 0.5 or 1 % (w/v) PINs or inulin. After 24 h of cultivation, the treated PA (2.0 x 10^5 CFU/ml) were co-cultured with *Salmonella* Gallinarum (2.0 x 10^5 CFU/ml) in MRS broth for 8 h at 37 °C with shaking (255 rpm). The protocol for co-cultivation assay previously described was followed to quantitatively compare the antimicrobial activity of PA cultures toward *Salmonella* Gallinarum. To examine whether the hydrophobic group was specifically required to enhance the antimicrobial ability, the antimicrobial activity of PA treated with acetyl inulin nanoparticles (AINs), propyl inulin nanoparticles (PrINs) was performed as described above.

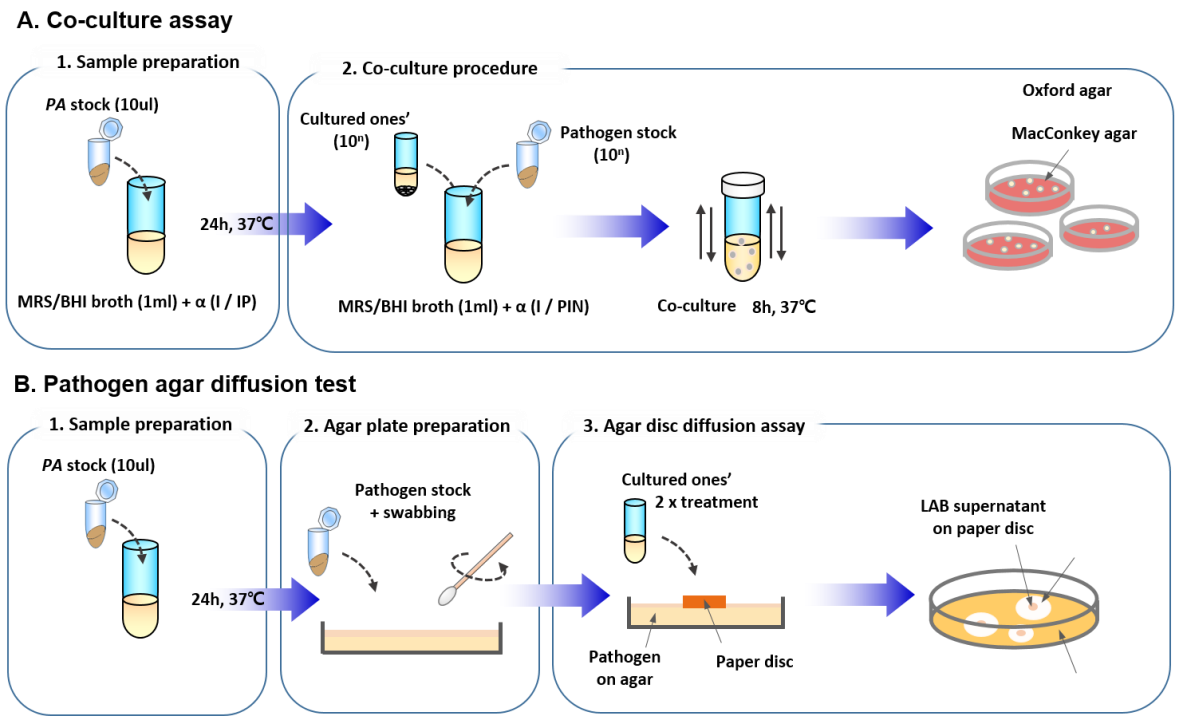


Figure 13. Procedure of measuring antimicrobial activity of PA. (A) Quantitative analysis for measuring antimicrobial activity of PA against pathogens. Viable cell counts of pathogen was measured using selective agar. (B) Agar diffusion test was used to determine cultured PA can inhibit the growth of pathogens. The zone of inhibition was measured.

6) Tracking the internalization of PINs in probiotics

Initially, fluorescence isothiocyanate (FITC)-labeled PINs were prepared. Briefly, 5 mg of FITC was mixed with 100 mg of PINs or inulin dissolved in 1 ml of dimethyl sulfoxide (DMSO). After stirring for 4 h in dark at room temperature, the reaction mixture was dropped into 10 ml of ethanol to remove the unreacted FITC. FITC-PINs or FITC-inulin were collected by centrifugation at 19,000 x*g* for 10 min. The fluorescence of the FITC-PINs or FITC-inulin was then quantified using a standard curve of FITC-mannan. For the encapsulation of curcumin, 20 mg of curcumin was mixed with 100 mg of PIN4 dissolved in 1 ml of DMSO. The mixture was dialyzed against distilled water at 4 °C for 24 h to form self-assembled PIN4/curcumin particles, which were finally lyophilized and stored at -20 °C until use.

To observe the size-dependent internalization of PINs in probiotics, PA (2.0 x 10^5 CFU/ml) were inoculated into 1 ml of MRS broth, treated with 0.1 % (w/v) FITC-PINs and incubated for 3 min at room temperature. After 3 min, the samples were washed with PBS and analyzed by flow cytometry and confocal laser microscopy (SP8 X STED, Leica, Wetzlar, Germany). To confirm the internalization of particles inside the probiotics, PA treated with FITC-PIN4 or PIN4/curcumin were analyzed by CLSM performed in Z-section mode.

To observe the temperature-dependent internalization of particles into probiotics, three separate cultures of PA were treated with 0.1 % (w/v) FITC-PIN4

and incubated at 4 °C, 25 °C and 37 °C for 6 h. The samples were further washed with PBS and analyzed by flow cytometry and confocal laser microscopy. To observe the transporter-dependent internalization of particles into probiotics, glucose, galactose and fructose were used as blocking agents. PA (2.0 x 10^5 CFU/ml) were inoculated into 1 ml of PBS and treated with 10 % (w/v) glucose, galactose and fructose for 10 min at 37 °C before treatment with 0.1 % (w/v) FITC-PIN4. After 6 h of incubation at 37 °C, the samples were washed three times with PBS, and the internalization of PIN4 was analyzed by flow cytometry and confocal laser microscopy.

7) Observation of probiotics by FESEM

Samples for observation by SEM were prepared by following the method described by Zeitvogel *et al*.(Fabian Zeitvogel 2016). Briefly, pre-fixation was performed with Karnovsky's fixation for 4 h, followed by three washes with 0.05 M sodium cacodylate buffer. Post-fixation was performed with 2 % osmium tetroxide and 0.1 M cacodylate buffer for 2 h. After washing 2 times with distilled water, dehydration was performed using a series of graded ethanol solutions (30, 50, 70, 80, 90 and 100 % ethanol in water). After dehydration, the samples were dried overnight using hexamethyldisilazane. Prior to SEM analysis, the samples were coated with Pt using an EM ACE200 (Leica, Austria) at 23 mA for 100 s and observed using an SEM (SUPRA 55VP, Carl Zeiss, Germany).

8) Observation of probiotics by TEM

Samples for observation by TEM were prepared by following the method described by Schrand *et al*.(Schrand et al. 2010). Fixation was performed using Karnovsky's fixation for 4 h, followed by three washes with 0.05 M sodium cacodylate buffer and post-fixation with 2 % osmium tetroxide and 0.1 M cacodylate buffer for 2 h. After washing two times with distilled water, the samples were dehydrated in a series of graded ethanol solutions (30, 50, 70, 80, 90 and 100 % ethanol in water). The pellet was then incubated in 2 ml of propylene oxide and 1 ml of Spurr's resin for 2 h, and then in 1 ml propylene oxide and 1 ml of Spurr's resin overnight. Next, a fresh batch of 100 % resin was added and cured at 60 °C for two days. After polymerization, the resin block was cut into 60–70-nm-thick sections using a Leica EM UC7 ultramicrotome. After staining with uranyl acetate, the samples were placed on 200 mesh copper grids, and images were obtained using a TEM (LIBRA 120, Carl Zeiss, Germany) operating at 120 kV.

9) Analysis of growth condition and SCFA production by PA

PA were treated with or without PINs or inulin as described above. The growth conditions for the PA were monitored by measuring the pH and viable cell counts at the indicated time points. To detect the production of SCFAs by gas chromatography (GC), the cultured supernatants were mixed with an internal standard (propionic acid-2,2-d2) and methanol. GC analysis was conducted according to a method described by Arokiyaraj *et al*.(Arokiyaraj et al. 2017). 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.

10) Isolation, purification and analysis of pediocin

Pediocin was isolated and purified as described previously(Barnali et al. 2014) with some modifications. PA were treated with or without PINs or inulin as described above. The cultures were centrifuged at 3,000 xg for 30 min at 4 °C, and the supernatants were stirred with ammonium sulfate (35 % v/v saturation) for 30 min. The precipitated proteins were obtained by centrifugation at 3,000 x g for 30 min at 4 °C. PBS buffer was added to dissolve the pellets, which were purified using a centrifugal filter from Sigma-Aldrich (St. Louis, MO, USA). The purified solutions were dialyzed against the buffer overnight. The dialyzed proteins were freeze-dried and stored at 4 °C for further analyses. While the protein concentration was determined by the Bradford assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe and compare the isolated pediocin with a standard pediocin. Pediocin was quantified using a standard curve of bovine serum albumin.

The specific activity of pediocin was determined as previously described(Ge et al. 2016) and was expressed as arbitrary units (AU) per ml. PA were treated with or without PINs or inulin as described above. Next, the culture supernatants were adjusted to pH 5.5 with 1 M sodium hydroxide to eliminate the antimicrobial effect of lactic acid. Pediocin activity was assayed by the agar well diffusion method and calculated based on the dilution ratio of the inhibitory activity. In brief, pediocin activity was determined by the diameter of the inhibition zone produced by PA (treated or untreated) that inhibited the growth of *Listeria monocytogenes*.

11) 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. Briefly, PA were treated with or without PINs or inulin as described above. After the isolation of RNA, 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 9 were designed based on the sequence reported by Fernandez *et al*.(Fernandez et al. 2014). For relative quantification, 0.01 ng of 16s rRNA cDNA was used when 1 ng of the *pedA, pedB, pedC* and *pedD* genes was used. 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 each sample. The data are presented as the relative fold-change compared with the probiotics control group.

Table 9. List of the primers used in this study

	Primer sequence (5'-3')	Size (bp)	
ped A	f:TGGCAAACATTCCTGCTCTGT	83	Structure
	r:CACCAGTAGCCCATGCCATAG		protein
ped B	f:ATTGCCAGCCAAGCGTTAGT	102	Immunity
	r:GCCCCACCCTTTTTGAGAAT		protein
ped C	f:CCATATCGGTGAG TGCTGACA	104	ABC
	r:AGGAATAACGCCCCTGATGTT		transporter
ped D	f:GGCCCATCTTCGACAGCTT	101	Regulatory
	r:GCACAGCTTCGGCATTTAAAT		protein
16S	f:GATGCGTAGCCG ACCTGAGA	113	
	r:TCCATCAGACTTGCGTCCATT		
dnaK	f:TTAACACGGGCACAATTTGA	212	
	r: GCTTCGTCAGGGTTAATGGA		
dnaJ	f: GCCCAACTTGTGGTGGTACT	240	
	r:CCAGTGCAGCTTGTACGAAA		
groEL	f:GGTAACGGTCGCGTTTTAGA	156	
	r:TTCAACGACTGCAACTAAGTCC		
groES	f:GGAAGACCTTGACGCAGAAG	239	
	r:CGTTTTGAAGTGCTGAACGA		
clpB	f:CGGCAGCCAAGTTATCTAGC	219	
	r:GCAGTGCCTTTAAGCGTTTC		

12) Sequencing and analysis of mRNA

For high-throughput sequencing, RNA was extracted at 24 h after treatment of PA with PIN4, and sequencing libraries were constructed using the TruSeq RNA kit (Illumina, CA, USA) according to the manufacturer's instruction. The prepared libraries were then sequenced using HiSeq 2500 (Illumina, CA, USA) for 100-bp paired-end reads. Adapter sequences of the reads were trimmed with Cutadapt1.10(Marcel 2011), and ribosomal RNA sequences were removed in silico using the SortMeRNA program(Kopylova et al. 2012). The sequence reads were quality-filtered using in-house Perl scripts(Kim and Marco 2014). In brief, when 95 % of the nucleotide bases in a read were given a quality score over 31 (Illumina 1.8+) and the read length was ≥70 bp, the read was used for transcript analysis. RNA-seq reads were mapped to the PA genome (NCBI accession MPJU00000000) using TopHat(Trapnell et al. 2009), and HTSeq was used to quantify the gene expression(Anders et al. 2015). EdgeR was used to quantify and normalize the gene expression(Anders et al. 2015). All programs were used with default options, and the gene expression level was normalized by fragments per kilobase of transcript per million fragments sequenced (FPKM). The genome of PA was annotated using rapid annotation subsystem technology (RAST) with default options, and all genes were categorized by this technology (Aziz et al. 2008). The mRNA sequences were registered in the NCBI Sequence Read Archive under accession SRR5411014.

For RNA extraction and quantification, the above-described protocols were followed. qRT-PCR was performed with SYBR qPCR Mix using one-step real-time PCR. All primers were designed using primer 3 software, and their sequences from 5' to 3' are shown in Table 9.

13) Hydrogen peroxide activity assay

PA (2.0×10^5 CFU/ml) was inoculated into 1 ml of MRS broth and treated with 0.5 % (w/v) of PINs or inulin. After incubation for 24 h at 37 °C with shaking (246 rpm), the pH of the culture supernatant was adjusted to 6. The culture supernatant was treated with 1 mg/ml catalase and incubated at 37 °C for 2 h. After incubation, the supernatant was heated at 100 °C for 30 min. Next, 5.0×10^7 CFU/ml of *Salmonella* Gallinarum were treated with 1 ml of the culture supernatant and incubated at 37 °C for 6 h with shaking (246 rpm). The *Salmonella* Gallinarum CFU for each culture supernatant were measured using MacConkey agar.

14) Statistical analysis

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

3. Results

1) Synthesis and characterization of PINs, AINs and PrINs

The reaction scheme of the synthesis of PINs is shown in Figure 14A. The morphologies of the PINs were spherical with nanometer sizes when observed using a SEM, and the number of smaller nanoparticles was greater in PIN4 as a consequence of the higher content of phthalic acid groups **(**Figure 14B). The degree of substitution of phthalate moieties in inulin was controlled by varying the molar ratio of phthalic anhydride to inulin, such as 0.3:1 (PIN1), 0.6:1 (PIN2), 1.2:1 (PIN3) and 2:1 (PIN4). The degree of substitution of phthalic groups in PINs was confirmed by ^{1}H-NMR spectroscopy (Figure 15A). The peak assigned to the protons of phthalic acid appeared at 7.4-7.7 ppm, and the peak assigned to the protons of inulin appeared at 3.8 ppm in the NMR spectra. Based on the integration of protons in phthalic acid and protons in inulin, the PINs were named as follows: PIN1 (content of phthalic acid: 9.9 mol.-%), PIN2 (content of phthalic acid: 15.2 mol.-%), PIN3 (content of phthalic acid: 20.4 mol.-%) and PIN4 (content of phthalic acid: 27.4 mol.-%). The sizes of the nanoparticles measured by DLS were 365, 330, 320 and 224 nm for PIN1, PIN2, PIN3 and PIN4, respectively, signifying that the particle sizes of PINs decreased in the following order (PIN1>PIN2>PIN3>PIN4) with an increase in conjugated phthalic acid groups in PINs (Figure 15B). Furthermore, the zeta-potentials of PINs measured by ELS were -21.29, -27.91, -26.34 and -23.64 mV for PIN1, PIN2, PIN3 and

PIN4, respectively (Figure 15C). The negative zeta potential arose due to the non-reacted carboxylic acids in the phthalic moieties of PINs that are deprotonated at pH 7 (distilled water).

For AINs and PrINs, the synthesis reaction scheme and their characteristics are shown in Figure 16**.** The morphologies observed by SEM, appeared to be nanoscale and the actual size of nanoparticles were measured by DLS were 552.4 nm (AINs) and 752.7 nm (PrINs). However, the polydivesity index were almost 0.7 in both AINs and PrINs. The conjugation ratio of hydrophobic moieties in AINs and PrINs was 78.8 mol.-% and 72.4 mol.-%. Protons of inulin appeared at 3.8 ppm, acetic acid at 2.0 ppm, and propionic acid at 1.0 ppm. Moreover, both nanoparticles showed negative zeta potential, although acetyl and propyl group does not contain carboxylic acids in the structure.

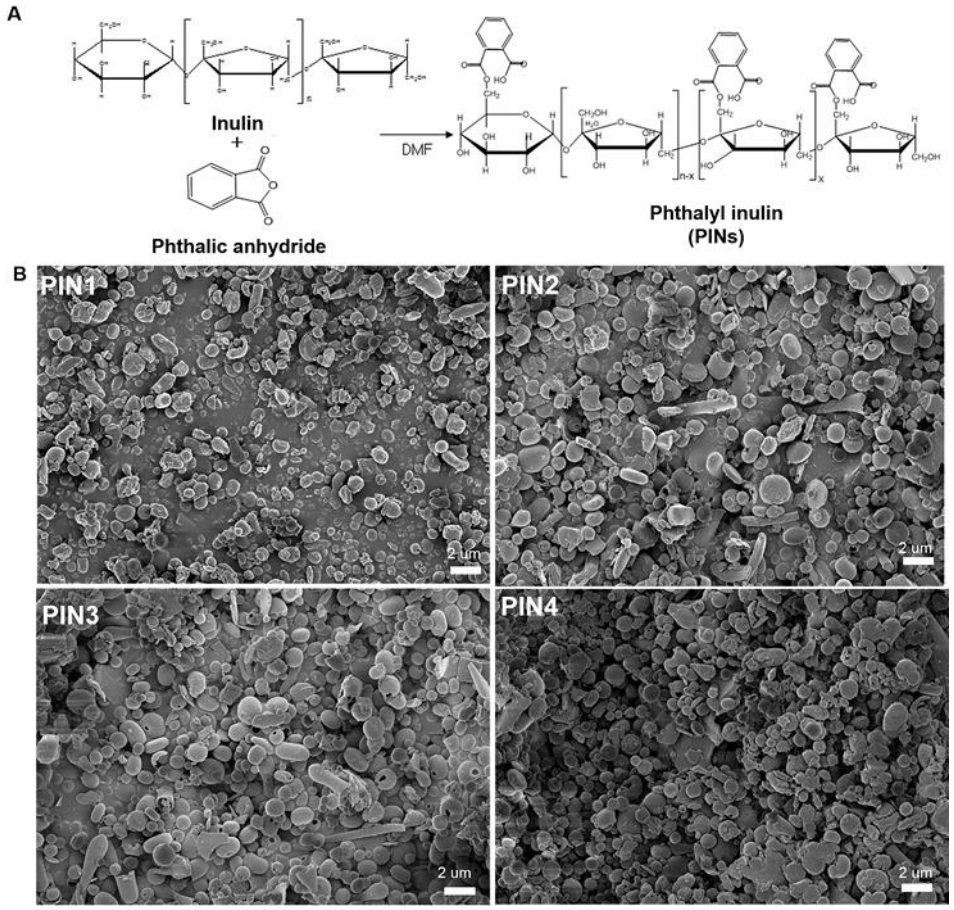


Figure 14. Chemical reaction scheme and morphologies of PINs. (A) Chemical reaction scheme for the synthesis of PINs. For the reaction in each PINs synthesis, the molar ratio between phthalic anhydride and inulin was as follows: 0.3:1 (PIN1), 0.6:1 (PIN2), 1.2:1 (PIN3) and 2:1 (PIN4). (B) Morphology of PINs observed by SEM. Magnification: 10,000X; Scale bar=2 μm.

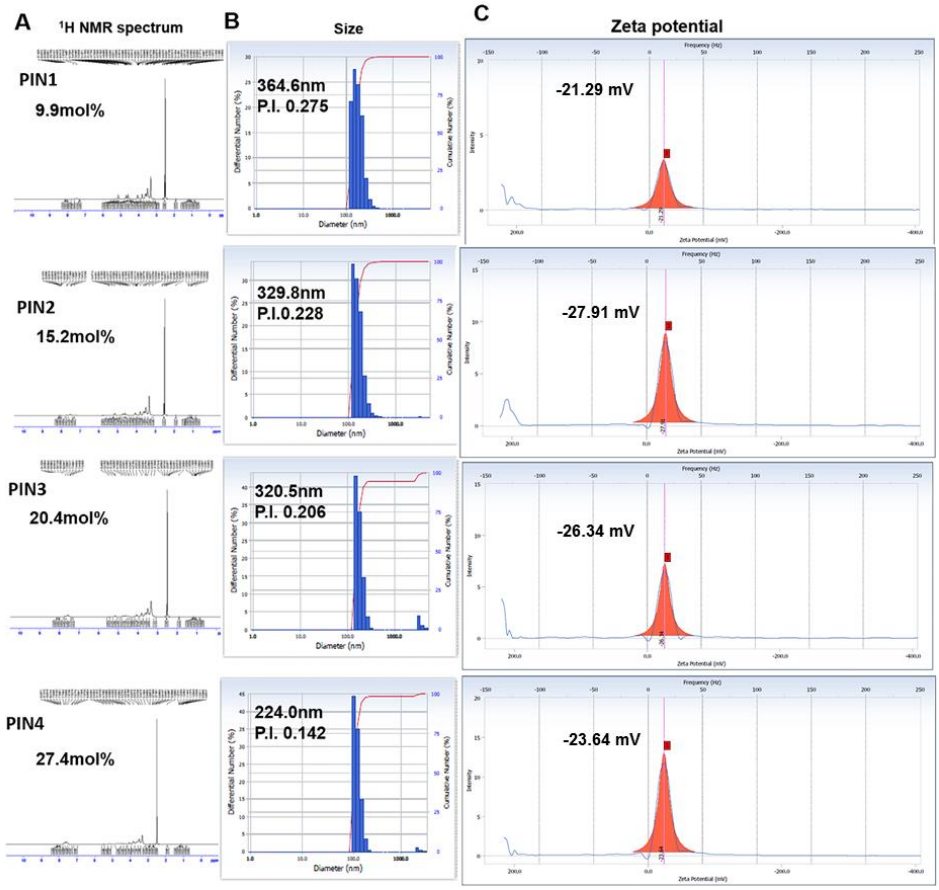


Figure 15. Characteristics of PINs. (A) Calculation of mol.-% of phthalic acid in PINs by ^{1}H-NMR spectroscopy. (B) Measurement of the sizes of PINs by DLS and (C) zeta-potential by ELS.

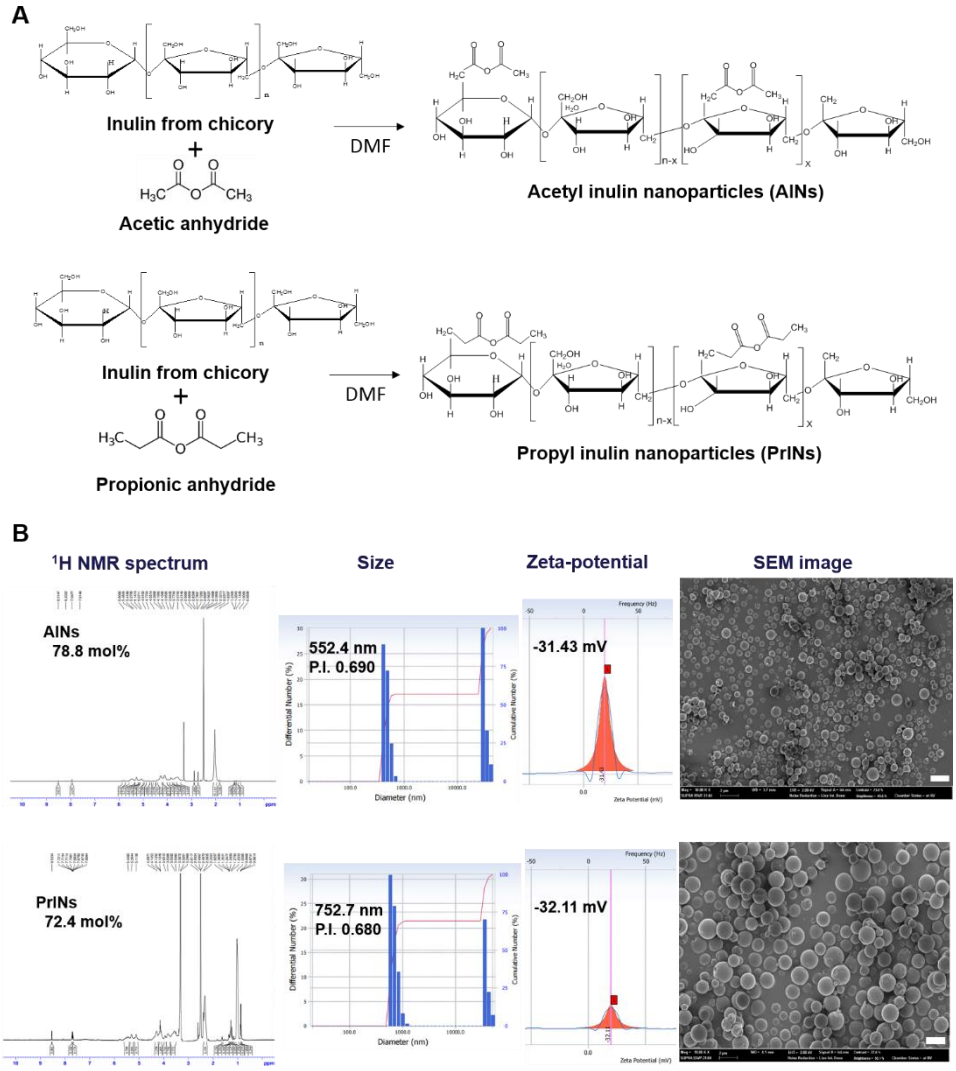


Figure 16. Chemical reaction scheme and characteristics of AINs and PrINs.

(A) Chemical reaction scheme for the synthesis of AINs and PrINs. The molar ratio was as follows: 1:1. (B) Characteristics of AINs and PrINs. Magnification: 10,000X; Scale bar=2 μm.

2) Effects of inulin nanoparticles on antimicrobial property of PA

To evaluate whether the treatment of nanoparticles to PA could affect the potency of antimicrobial peptide production, and to assess the specificity in the antibacterial properties of probiotics according to the nature of the hydrophobic group in inulin nanoparticles, PA was separately treated with three type of individual nanoparticles. The antibacterial potency induced by each PA treated with PINs (PA/PINs), PA treated with AINs (PA/AINs) and PA treated with PrINs (PA/PrINs) were tested against *Salmonella* Gallinarum (Figure 17). All types of nanoparticles could significantly enhance the antimicrobial property of PA, indicating that the induction of antimicrobial property in probiotics was not specific to the nature of hydrophobic groups in inulin nanoparticles. PA treated with PINs showed significantly higher antimicrobial activity against SG among other nanoparticles groups.

To see the more advanced antimicrobial activity with PA/PINs, PA/PINs was tested against Gram-negative *Salmonella* Gallinarum, ETEC K88 and Gram-positive *Listeria monocytogenes*. Compared with PA alone, treatment with each PA/PINs resulted in higher antibacterial activity against both *Salmonella* Gallinarum, ETEC K88 and *L. monocytogenes* in co-culture assays (Figure 18A, C, E). Interestingly, the antibacterial potential of PA increased with a decrease in particle sizes of the internalized PINs. Moreover, PA/PINs had relatively higher antibacterial activity than PA/I (soluble inulin) against both *Salmonella* Gallinarum, ETEC K88 and *L. monocytogenes*. To examine if the enhanced

antibacterial activity was induced by the PINs alone, *Salmonella* Gallinarum and *L. monocytogenes* was treated with PINs in the absence of PA. Only the PINs themselves had no antibacterial properties (Figure 19A), indicating that the antibacterial potential emerged from PA due to interactions with PINs. In addition, the antibacterial potential of PA in the presence of PINs was tested against *Salmonella* Gallinarum, ETEC K88 and *L. monocytogenes* by agar diffusion test (Figure 18B, D, F). The results of the agar diffusion test were evaluated by measuring the diameter of the zone of inhibition produced as a direct consequence of antimicrobial peptide production by PA/PINs. Consistent with the results of the antimicrobial test, the agar diffusion tests also showed similar pattern of antibacterial activity of PA/PINs against *Salmonella* Gallinarum, ETEC K88 and *L. monocytogenes*. Again, the zone of inhibitions was relatively larger when the PA was internalized smaller PINs.

To determine an optimum concentration of PINs to induce the antibacterial potential of PA, different concentrations of PINs or inulin were applied to PA. While the treatment with 1.0 % (w/v) PINs induced the antimicrobial potential of PA comparably higher than the treatment with 0.5 % (w/v) PINs, there were no significant differences between the antimicrobial potentials of PA at these concentrations (Figure 19B). Hence, 0.5 % (w/v) PINs was chosen to induce PA in the antimicrobial activity assay for further study.

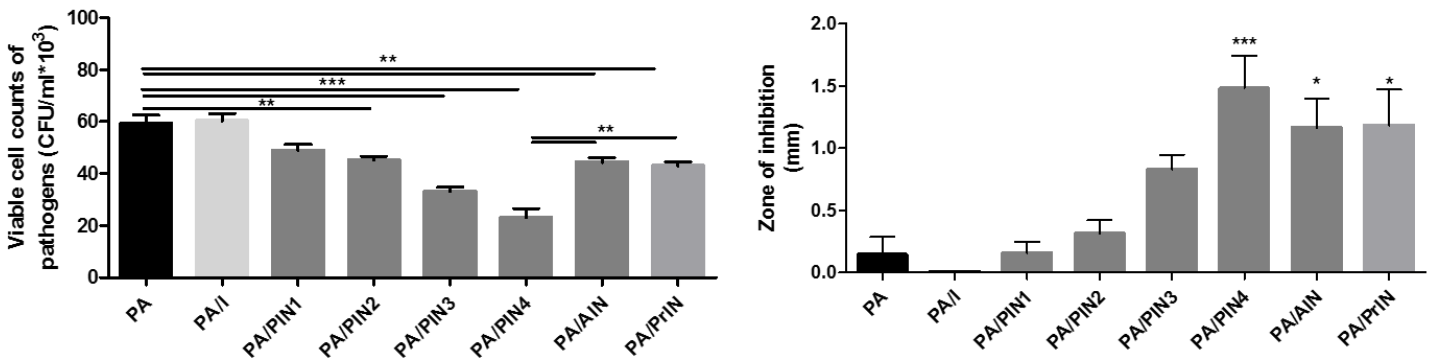


Figure 17. Antimicrobial activity of PA against pathogens in different conditions. Antimicrobial activity of PA after treatment with or without PINs, AINs and PrINs against *Salmonella* Gallinarum. The viable cell and diameters of the growth inhibition of pathogen by PA was measured.

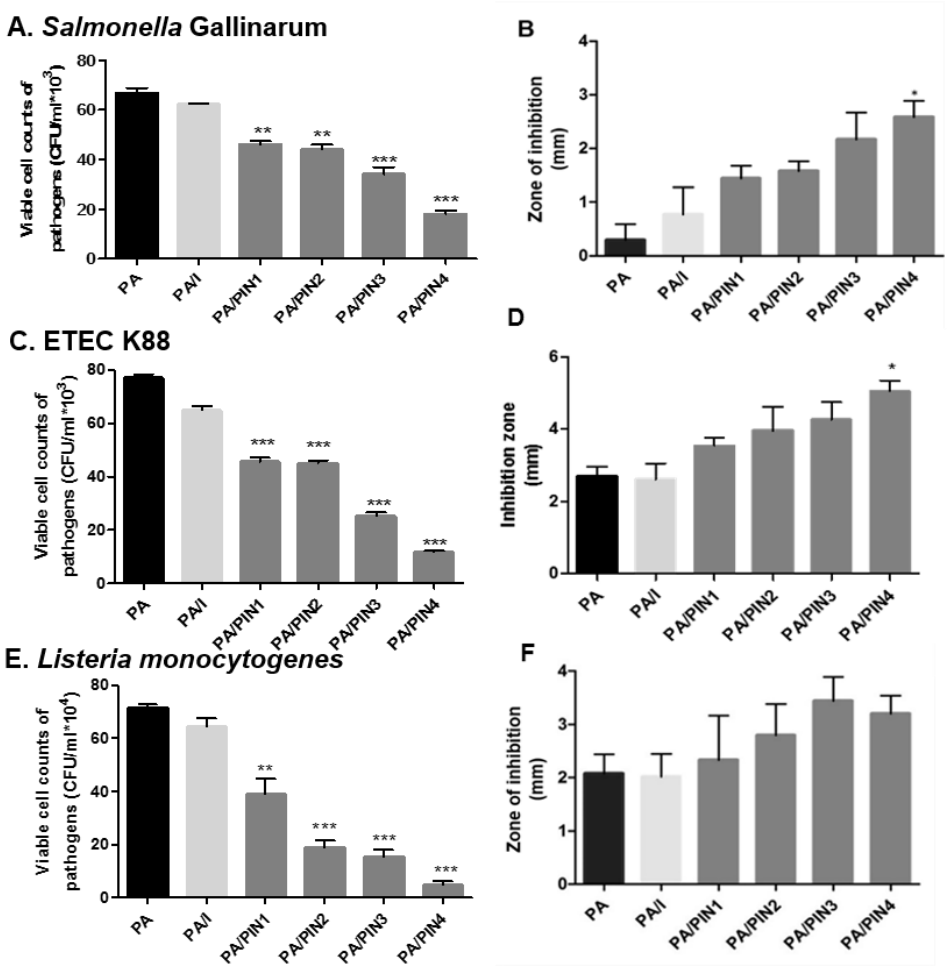


Figure 18. Antimicrobial efficacy of PINs-treated probiotics against pathogens. (A,C,E) PA treated with PINs or inulin were cultured with Gram-negative *Salmonella* Gallinarum, ETEC K88 or Gram-positive *L. monocytogenes* and the growth inhibition was calculated by CFU. (B,D,F) Similarly, the diameters of the growth inhibition of pathogens on agar plates was measured.

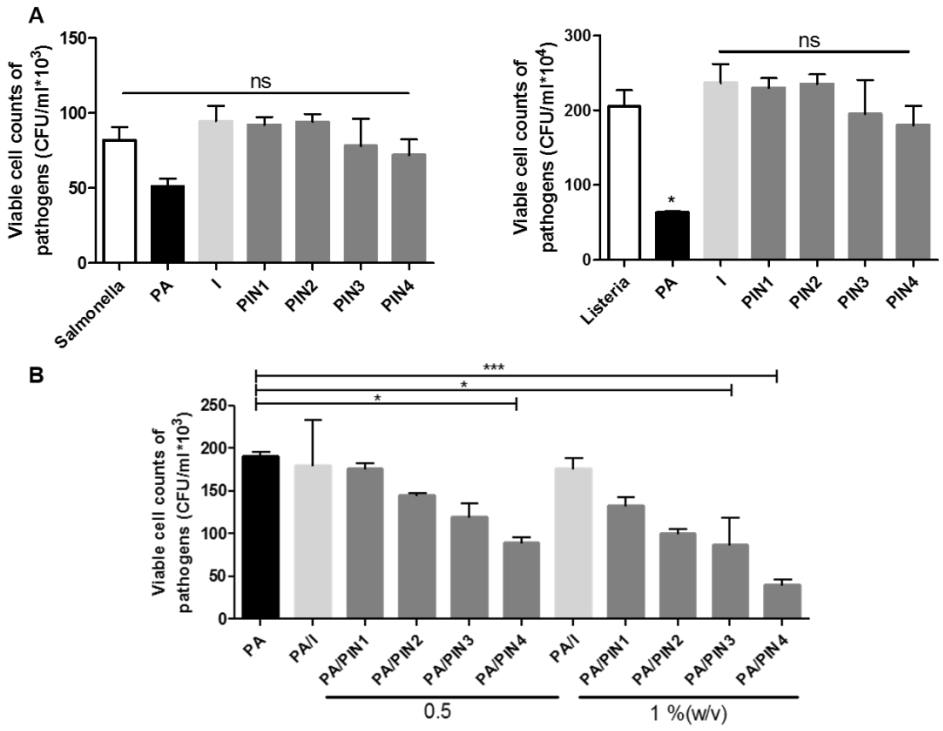


Figure 19. Antimicrobial activity of PINs and in different concentration. (A) Antibacterial activity on *Salmonella* Gallinarum and *L. monoctogenes* treated with PINs in the absence of PA. (B) The growth inhibition of *Salmonella* Gallinarum with different concentration of PINs

3) Internalization of PINs into probiotics

To study the internalization of PINs into probiotics, PINs were conjugated to fluorescence isothiocyanate (FITC), and *Pediococcus acidilactici* (PA) was used as a probiotic strain. The internalization of FITC-PINs into PA was analyzed by confocal laser scanning microscopy (CLSM) and quantified by fluorescence-activated cell sorting (FACS). Initially, in CLSM images FITC-inulin was able to enter into PA within 3 min of incubation at room temperature (Figure 20A). Similarly, internalization of PIN1, PIN2, PIN3 and PIN4 into PA was observed within 3 min of incubation at room temperature. Among the PINs, the highest internalization rate was observed for PIN4 (33.3 %), while the internalization of PIN1 was only 0.61 % (Figure 20A, B). These results demonstrated that the internalization of PINs into PA increased with the decrease in particle sizes of PINs, suggesting a size-dependent internalization of PINs. Hence, unless otherwise stated, further experiments were selectively performed by treating PA with PIN4 only.

To further examine whether the PINs were on the surface or inside PA, PA were treated with FITC-PIN4, and CLSM was performed in Z-section mode. As shown in Figure 21A, the fluorescence intensity was highest at the center of PA, indicating the internalization of PIN4 into PA. In an alternative method to observe the internalization of PIN4 into PA, PIN4/curcumin was used to treat PA and observed the uptake by CLSM performed in Z-section mode. The result also showed that PIN4/curcumin were embedded in PA (Figure 21B).

To observe the morphological differences in PA due to the internalization of PINs, PA treated with or without PINs was observed using an energy-filtered transmission electron microscope (TEM). TEM images could not distinctly locate the PINs inside the PA **(**Figure 22**)**. In fact, the internal compartments of PA appeared dark in the TEM images due to the thickness of the PA. Moreover, the morphology of the PA treated with or without PIN4 by SEM to evaluate any structural changes in PA. The images demonstrated that PIN4-treated PA was indistinguishable from untreated PA **(**Figure 23**).**

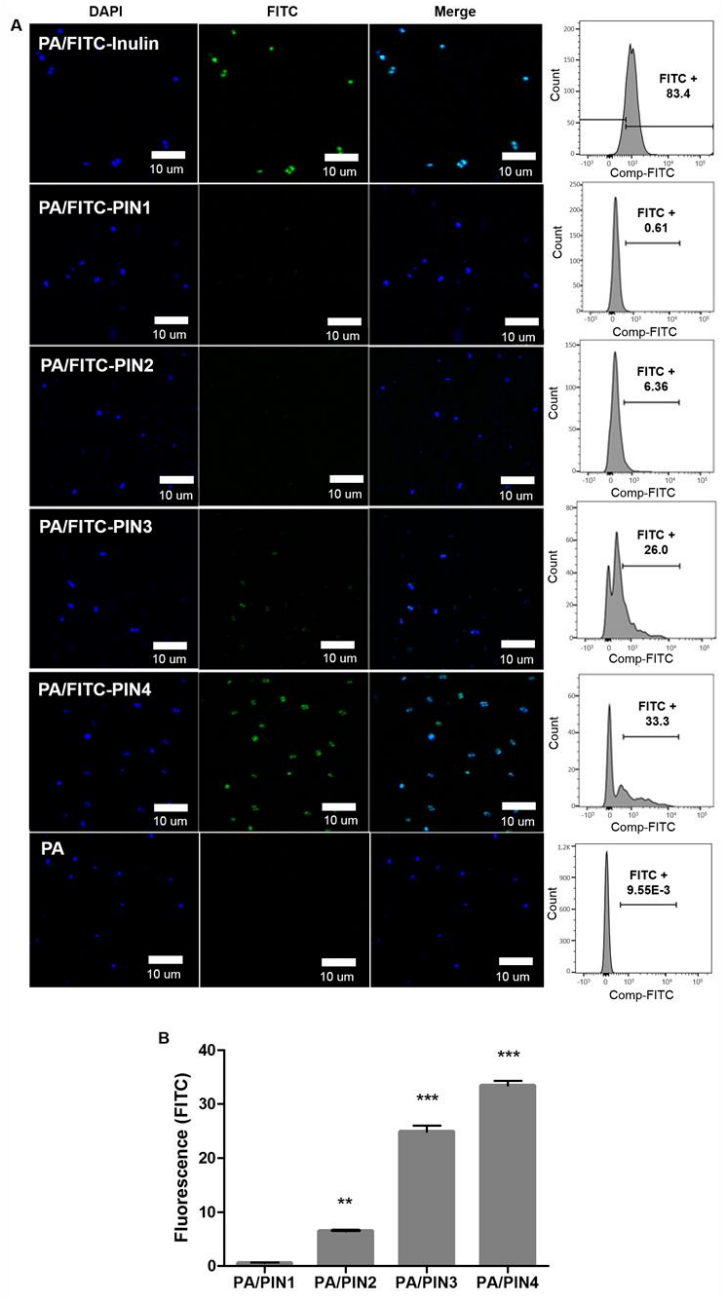


Figure 20. Analysis of the internalization of PINs in PA. (A) Confocal images and FACS analysis were performed after treatment of PA with 0.1 % (w/v) FITC-

PINs or FITC-inulin for 3 min at room temperature. FITC-PINs or FITC-inulin are shown in green, and PA was stained blue with DAPI. (B) The internalization of PINs after 3 min of treatment was quantified by FACS and statistically analyzed. Confocal and FACS data are representative of three independent experiments, and the average values are presented as the mean ± SEM of three independent FACS experiments by a bar chart.

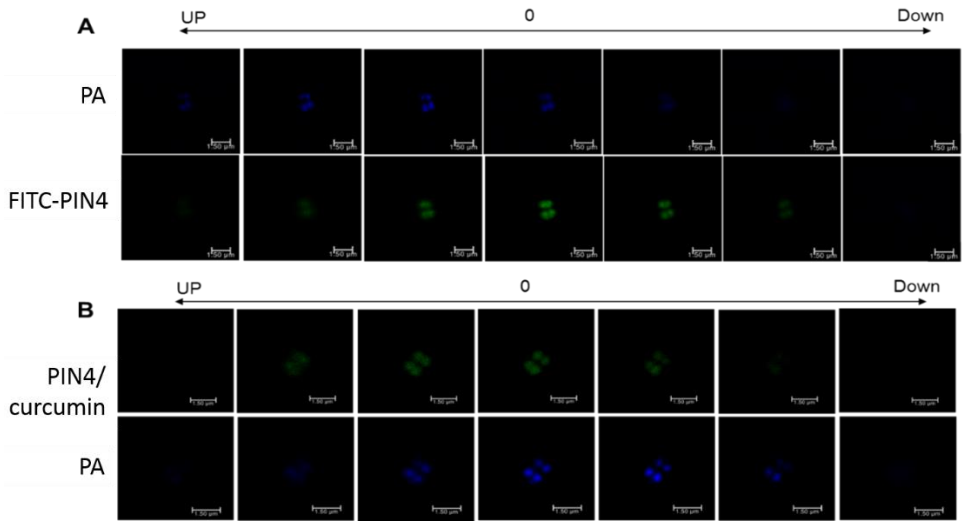


Figure 21. Analysis of the internalization of PINs into PA by Z-section mode. Confocal images were obtained after treatment of PA with (A) FITC-PIN4 and (B) PIN4/curcumin for 3 min at room temperature. Z-section images show the internalization of corresponding PINs into PA. FITC-PIN4 or PIN4/curcumin is shown in green, and PA was stained blue with DAPI. Scale bar=1.5 μm.

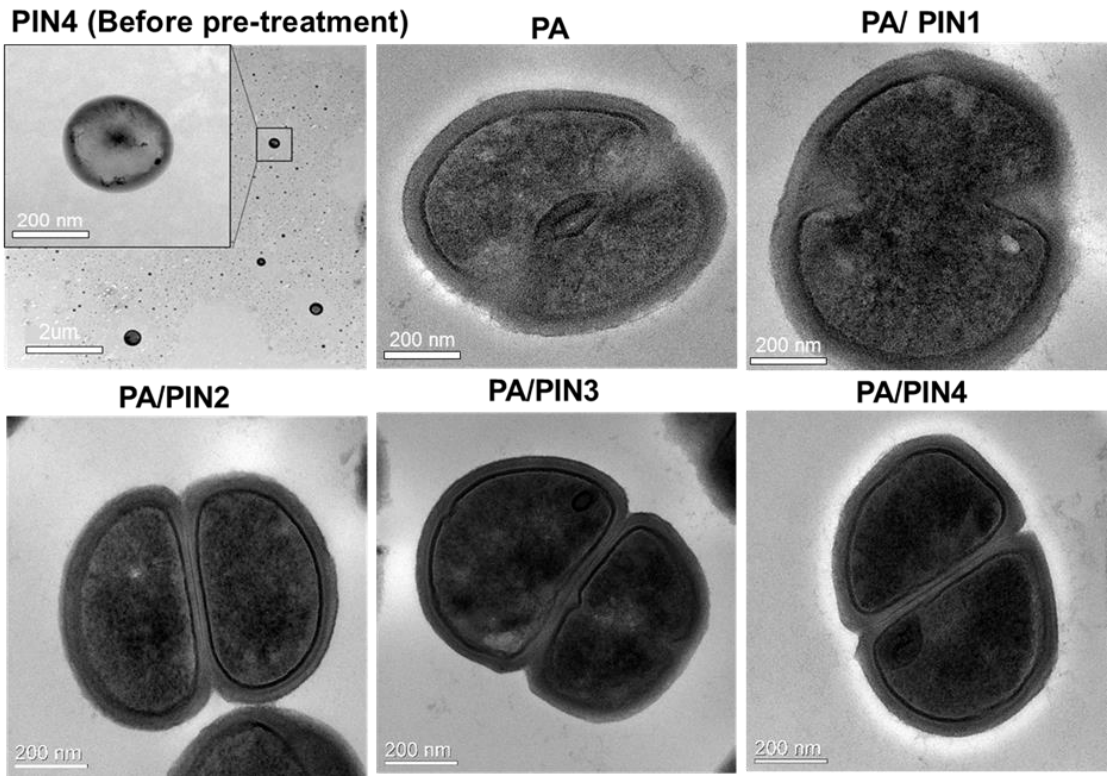


Figure 22. Analysis of the internalization of PINs by TEM. PA were treated with PINs for 24 h and analyzed by TEM to visualize the internalization of PINs into the probiotics.

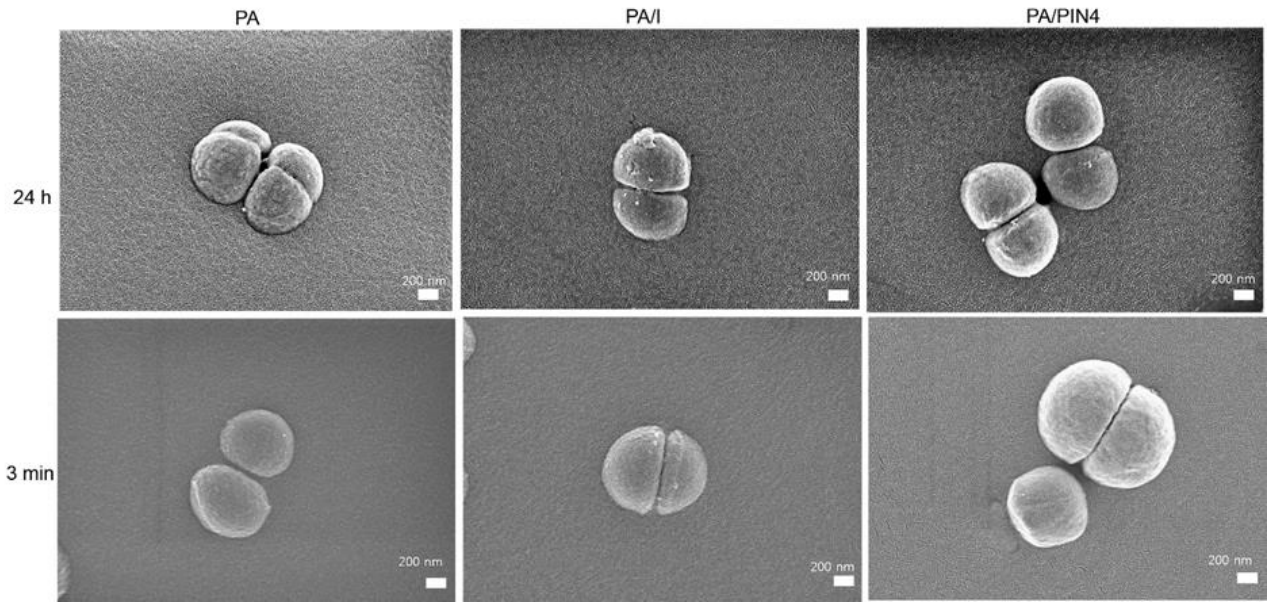


Figure 23. Morphology of PA with or without treatment. PA were treated with inulin (I) or PIN4 for 3 min or 24 h and analyzed by SEM to visualize any structural changes in PA.

Further studies were performed to examine the internalization of PINs into PA according to the incubation temperature and transporters in PA. To check the temperature-dependent internalization of PINs into PA, FITC-PIN4 was treated with PA at 4, 25 or 37 °C for 6 h, and subsequently analyzed by CLSM and FACS (Figure 24A). The results showed that the internalization of PIN4 into PA was significantly higher at 37 °C than 4 °C, suggesting an energy-dependent internalization of PINs. The results also indicated that PIN4 was more able to be internalized by PA at microbial growth than other temperatures.

Furthermore, a study was conducted to assess whether PINs were specifically internalized by transporter-mediated internalization. Typically, probiotics, pre-incubated with 10 % (w/v) glucose, fructose or galactose, was treated with 0.1 % (w/v) FITC-PIN4 for 6 h, and internalization was observed by CLSM and FACS. The results showed that the internalization of PINs was variably dependent on the specific transporter (glucose, fructose and galactose) (Figure 24B). Pre-treatment with glucose impeded approximately 42.6 % of PIN4 internalization, whereas pretreatment with galactose and fructose impeded 27.6 % and 15.5 % of PIN4 internalization, respectively. Overall, PIN internalization was significantly more retarded in the presence of glucose compared with fructose, suggesting that glucose transporters play a substantial role in PIN internalization.

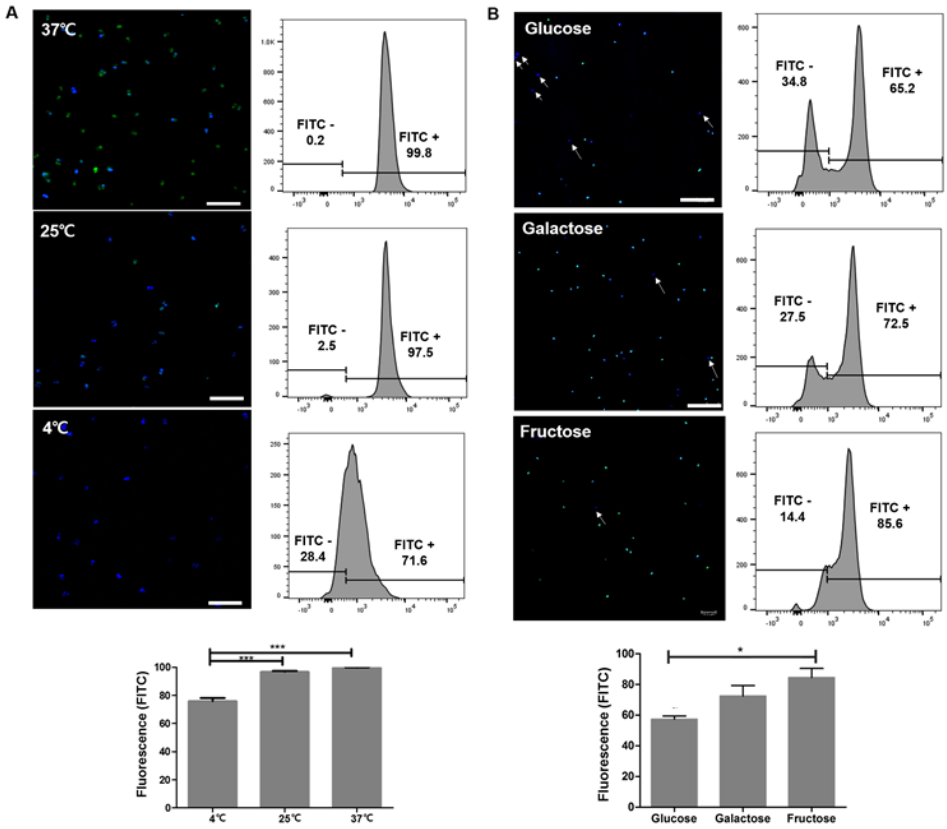


Figure 24. Analysis of the internalization of PINs in PA depending on temperature and transporters. (A) PA was treated with 0.1 % (w/v) FITC-PIN4 at different temperature for 6 h, and internalization was observed. (B) PA pre-incubated with 10 % (w/v) glucose, fructose or galactose, was treated with 0.1 % (w/v) FITC-PIN4 for 6 h at 37 °C, and the internalization was observed. FITC-PIN4 is shown in green, and PA was stained blue with DAPI.

4) Effects of PINs on growth, pH and SCFA production of PA

To observe the changes in the growth conditions of PA after treatment with PINs or inulin, viable cells were counted at different time intervals (Figure 25A). The results of the PA growth curve with or without PIN or inulin treatment showed no remarkable differences in PA growth. The pH of the culture medium of PA after treatment with PINs or inulin was also measured to evaluate the changes in lactic acid production (Figure 25B). Consistent with the growth curve, the pH curve of the PA with or without treatment with PINs or inulin also showed no significant changes in the pH of the culture medium among the groups. The results indicated that internalization of PINs or inulin had no effect on the normal growth of PA.

To further examine any internal changes in PA by PINs, PA was treated with PINs or inulin, and commonly secreted products from PA such as SCFAs were analyzed. The results indicated that total SCFA contents in the culture medium of PA decreased after the treatment with PINs or inulin (Figure 25C). The amount of total SCFA production in PA appeared to depend inversely on the particle sizes of internalized PINs. The greater the internalization of smaller PINs into PA, the less the production of SCFAs in PA. Although the production of SCFAs was comparably lower in PA by PINs than inulin, there were no significant differences among the treatment groups.

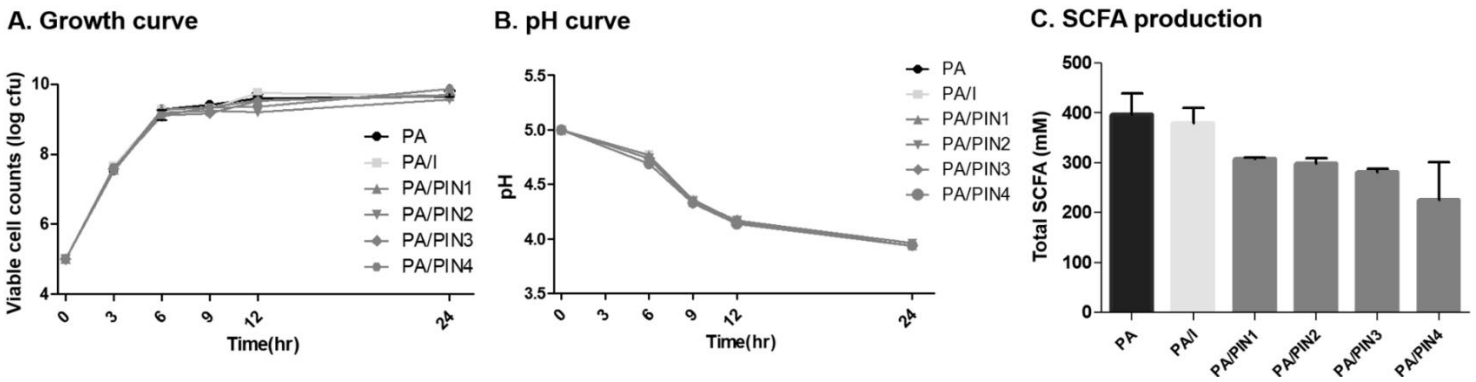


Figure 25. Analysis of biological effect of PINs in probiotics. (A, B) Measurement of the growth of PA and pH of the culture medium with or without PINs or inulin treatment. (C) Total SCFA contents in the culture medium of PA treatment with PINs or inulin

5) Effects of PINs on pediocin production by PA

To determine the variations in the production of pediocin in PA by PINs, the pediocin from PA, PA/PINs and PA/I was isolated, confirmed and quantified by cell lysis, SDS-PAGE and the Bradford assay, respectively. First, molecular weight of isolated pediocin was confirmed by SDS-PAGE using a standard pediocin as a reference (Figure 26A). The results showed that the molecular weight of isolated pediocin was approximately 3.5 kDa. Additionally, SDS-PAGE showed that PA/PINs showed increased production of pediocin compared with the PA group under the same isolation conditions. Furthermore, the isolated pediocin from each PA with or without treatment was quantified by the Bradford assay. Compared with PA, PA/PINs and PA/I showed significantly higher production of pediocin (Figure 26B). The results revealed that the production of pediocin was 4-fold higher in PA/PIN4 than PA alone. Similarly, the specific activity of the isolated pediocin was measured by the arbitrary unit. Consistent with the results obtained for pediocin production, PA/PINs and PA/I showed significantly higher pediocin activity than PA alone. Particularly, the activity of pediocin was significantly higher (9-fold) in PA/PIN4 compared with PA (Figure 26C). Altogether, the production of pediocin in PA/PINs also increased with a decrease in the size of internalized PINs into PA.

To evaluate the variations of pediocin production in PA at the genetic level, a study was undertaken to compare the gene expression profiles of pediocin biosynthetic genes using quantitative real-time PCR (qRT-PCR) (Figure 26D).

Four pediocin genes (*pedA, B, C and D*) were selected, and 16s rRNA were used for normalization. Following 24 h of treatment of PA with PINs or inulin, the relative gene expression of *pedA* was substantially higher in PA/PINs or PA/I than PA. Similarly, the expression level of *pedD* was variably higher in PA/PINs or PA/I than PA. In both cases, there were significant differences in the expression levels of *pedA* and *pedD* in PA/PIN4 compared with PA. However, the level of *pedC* expression showed no differences among groups, and the level of *pedB* expression was too low to detect by qRT-PCR. These gene profile data clearly revealed the variations in the expression levels of pediocin biosynthetic genes in PA when internalized to PINs. In contrast, there was no significant difference in the production of hydrogen peroxide (H_2O_2) in PA/PINs compared with PA or PA/I **(**Figure 27**).**

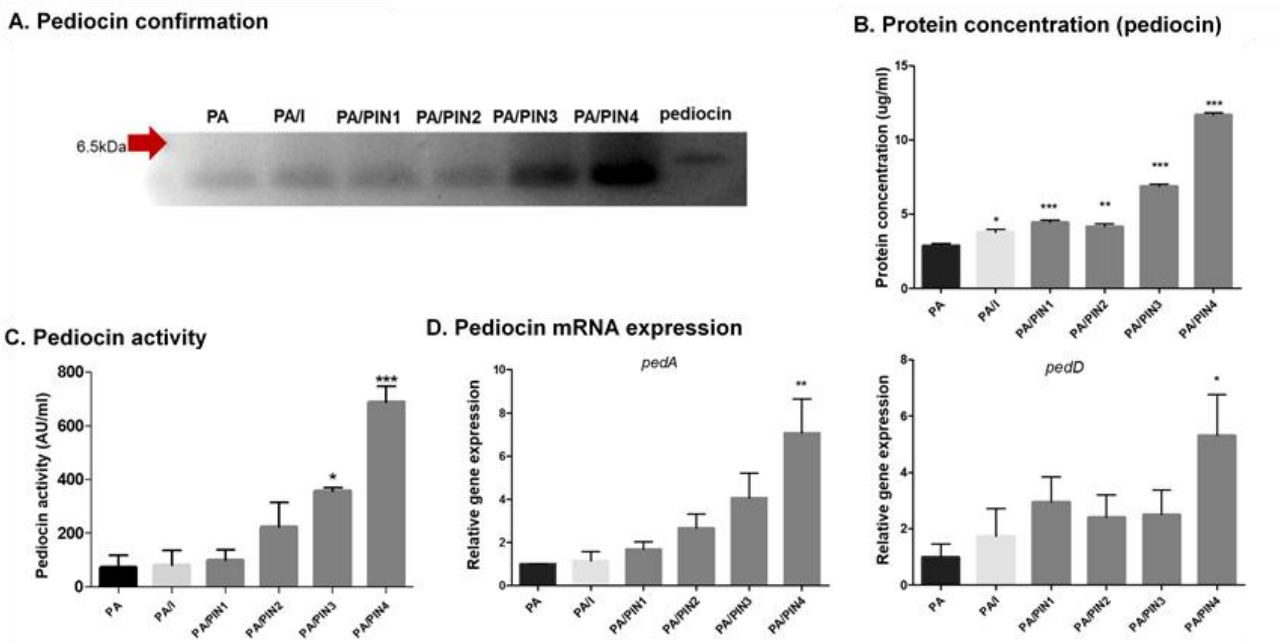


Figure 26. Analysis of the production of pediocin in PA by PINs. (A) The molecular weight of pediocin was determined by SDS-PAGE using a reference pediocin. (B) Quantification of pediocin by the Bradford assay. (C) Determination of the activity of pediocin (AU/ml) by measuring the diameter of the inhibition zone produced by PA (treated or untreated) against the growth of LM. (D) Relative mRNA expression of *pedA* and *pedD* compared with 16S rRNA expression.

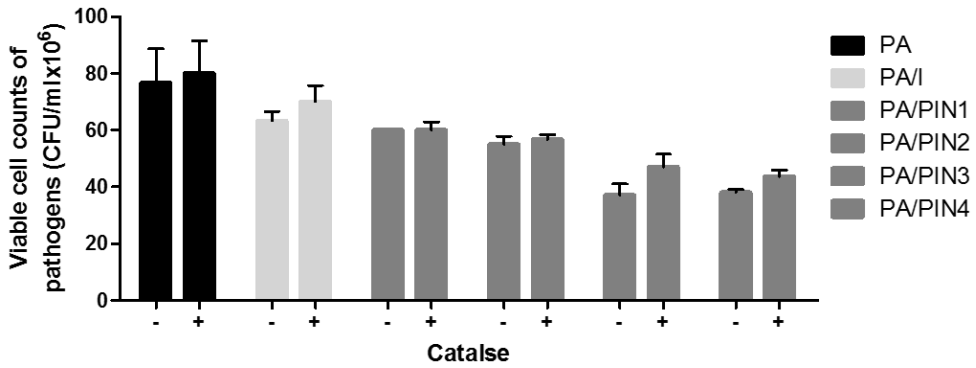


Figure 27. Hydrogen peroxide activity assay. Growth of *Salmonella* Gallinarum culture supernatants: +: catalase treatment, -: no catalase treatment. Each experiment was performed in triplicate, and each point represents the relative mean value.

6) Effects of PINs on the transcriptome of PA

To analyze the patterns of gene expression alterations in PA with or without PIN4, high-throughput sequencing was performed to determine the mRNA expression levels. The sequencing results revealed several changes in the number of differentially expressed genes (DEGs) in PA/PINs. There were 930 DEGs among a total of 2,125 genes in the genome ($p<0.05$). Among the DEGs, the expression levels of 31 genes were increased 1.5-fold in PA/PINs compared with PA, whereas the expression levels of 61 genes were decreased 1.5-fold in PA/PINs (Figure 28A, B). The genome of PA was annotated using rapid annotation subsystem technology (RAST), and the genes were categorized based on the variations in expression levels. As shown in the figure, the expression level of genes for RNA metabolism, DNA metabolism, and cell wall and capsule increased, whereas transcripts belonging to the carbohydrate category decreased (Figure 28C). Interestingly, the expression level of several genes (*groEL, groES, dnaK, dnaJ and clpB*) related to heat shock proteins of PA with internalized PINs increased significantly, suggesting that the internalization of PINs into probiotics led to changes in the expression of genes involved in the stress response (Figure 28D). Hence, the levels of gene expression related to the stress response, i.e., molecular chaperones (*groEL, groES and dnaK, dnaJ)* and Clp protein (*clpB*), were further analyzed by qRT-PCR. Changes in the transcription level of *groEL*, *groES*, and *dnaK* in PA/PIN4 were statistically significant, showing 5.2, 4.6 and 4.4-fold increases, respectively, compared with PA alone (Figure 29A-C). The

transcription of *dnaJ* and *clpB* also increased 3.3 and 6.7-fold, respectively in PA/PIN4 compared with PA (Figure 29D-E). Specifically, the expression levels of heat shock proteins in PA increased with the decrease in particle size of internalized PINs, suggesting that the greater the internalization of PINs into PA, the stronger is the induction of stress responses.

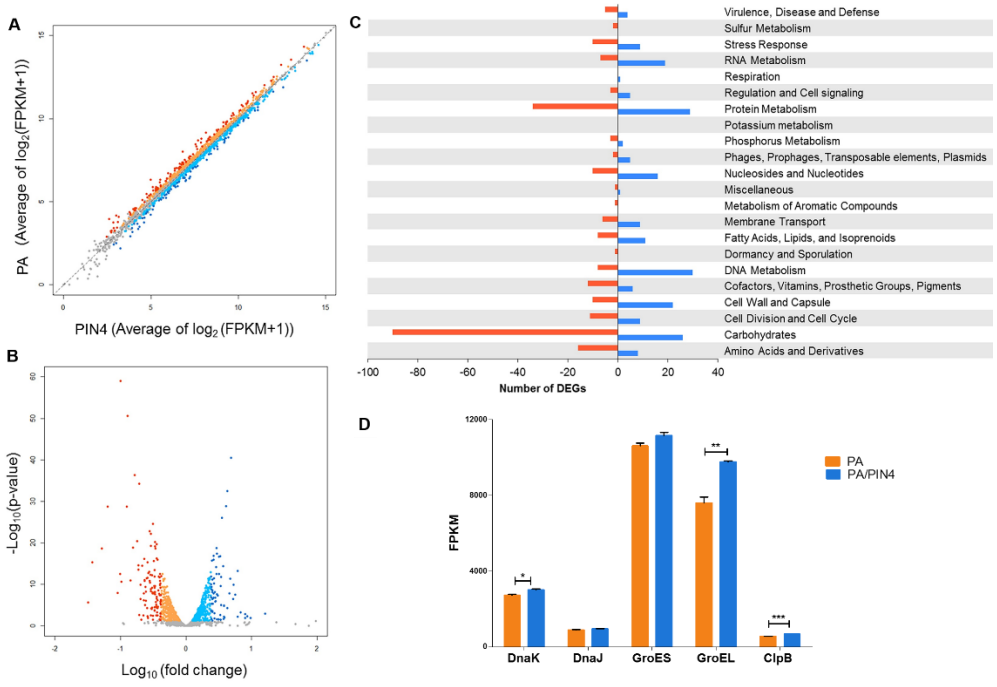


Figure 28. Distribution of differentially expressed genes (DEGs) between PA and PA with internalized PIN4 ($p < 0.05$). (A) Scatter plot representing the distribution and expression levels of the examined genes and (B) volcano plot representing the statistical significance with respect to PIN4 are presented. Red dot, PA-preferential gene (fold change ≥ 1.5); yellow dot, PA-preferential gene (fold change < 1.5); blue dot, PIN4-preferential gene (fold change ≥ 1.5); azure dot, PIN4-preferential gene (fold change < 1.5); gray dot, non-DEG. (C) Functional categorization of the DEGs with RAST. (D) Gene expression related to stress response

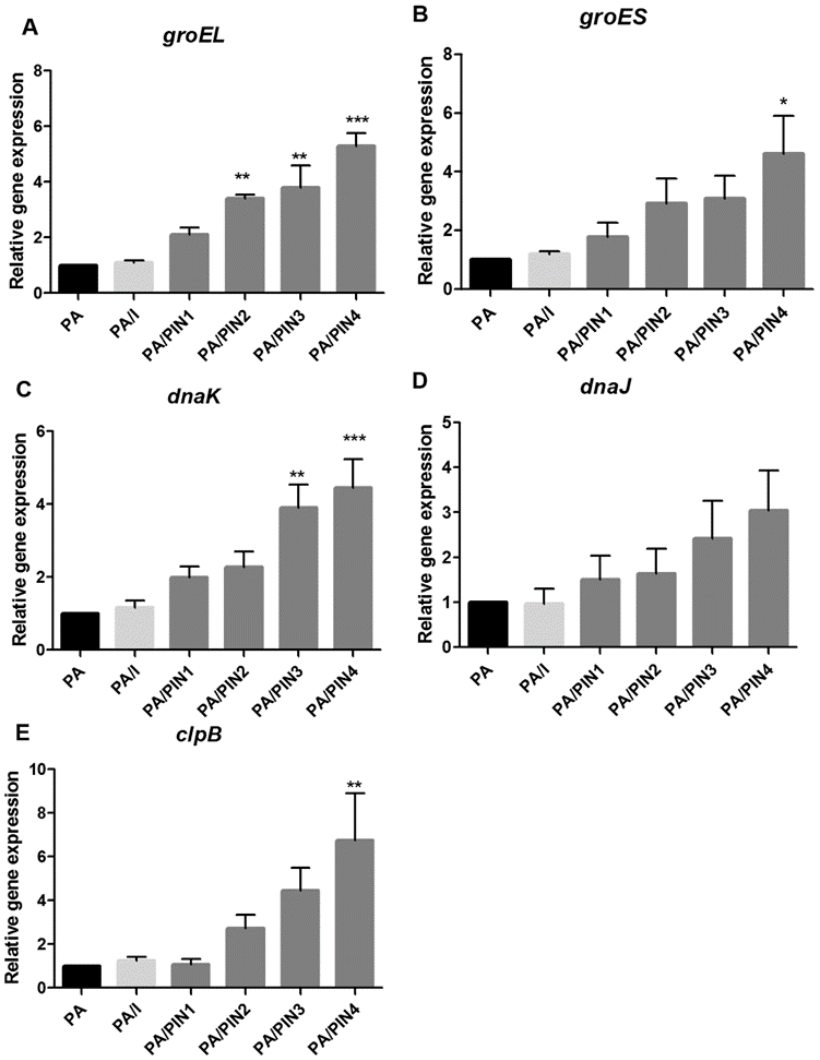


Figure 29. Analysis of gene expression related to the stress response in PA treated with PINs. The transcriptional expression of *groEL* (A), *groES* (B), *dnaK* (C), *dnaJ* (D) and *clpB* (E) relative to 16S rRNA was quantified by qRT-PCR.

4. Discussion

In study 1, as a new formulation of prebiotics, PINs, AINs, and PrINs were developed to enhance the antimicrobial potential of probiotics. Prebiotic nanoparticles (PINs, AINs, and PrINs) were easily made by a self-assembly method. Phthalyl anhydride, acetic anhydride, and propionic anhydride were conjugated to inulin as hydrophobic groups to hydroxyl groups in inulin through hydrophobic interactions. It is assumed that the reaction was occurred through an esterification mechanism between the primary hydroxyl group of the inulin and carboxylic acids of phthalic anhydride after the ring opening, since primary alcohols are more reactive than secondary ones. For AINs and PrINs, methyl group of acetic anhydride and propionic anhydride was conjugated with hydroxyl group of the inulin. Moreover, possibly due to the enhanced hydrophobic interactions between phthalic moieties, a higher conjugation rate of phthalic groups in PINs could result in smaller particle sizes and spherical shapes of PINs. However, AINs and PrINs had higher conjugation rate of hydrophobic moiety, the sizes were larger than PINs.

The study of the internalization of prebiotics into prokaryotes is still in an early stage so far. A large amount of research has been focused on how foreign nanoparticles are internalized into eukaryotic cells through endocytosis (Oh and Park 2014). Most research on prebiotic materials have mainly focused on fermentation by probiotics (Ji-Lin et al. 2017; Valdes et al. 2017). By contrast,

developing prebiotics in nanoparticles form and elucidating their internalization into probiotics was one of the goals in this study. Since it has been reported that soluble prebiotics can enter probiotics by passive diffusion through transporters (Barrangou et al. 2006; Saulnier et al. 2007)[,]. Moreover, by far, the internalization of nanoparticles into bacteria is only reported with metal nanoparticles. It has been reported that metal nanoparticles are internalized into *E. coli* via electrostatic interactions(Sanyasi et al. 2016). However, in this study it was able to assume that PINs would enter PA through transporters such as carbohydrate receptors at the cell surfaces of probiotics (PA). In support of this assumption, pretreatment with glucose significantly decreased the internalization rate of PIN4 into PA, while galactose and fructose inhibited the internalization to a reduced extent. These results revealed that the glucose units in the inulin backbone of PIN4 are preferably recognized by glucose transporters in PA to assist the internalization of PIN4.

For the main goal of the study, which prebiotic nanoparticles can effect the probiotics in cellular and antimicrobial properties, PINs, AINs and PrINs were treated to PA. The treatment of PA with PINs greatly increased antimicrobial activity against both Gram-negative and Gram-positive pathogens compared with the treatment with inulin or AINs or PrINs or PA itself. Moreover, the PINs used in this study did not show any toxicity toward PA, but rather they enhanced the production and activity of pediocin. Although the mechanism is not clear, from the transcriptional assay, it seems that internalization of PINs by PA causes mild

stress to activate the probiotic defense system, leading to increased production of pediocin. In particular, PA/PIN4 exhibited the highest antibacterial activity and pediocin production. These results indicated that as the internalization of small-sized particles into PA was higher, it appeared that the increased antimicrobial activity of PA was dependent on the amount of particles taken up by PA.

Probiotics are widely used in food and feed additives for their beneficial roles, such as immunomodulation, modulation of intestinal microflora, prevention of diarrhea, and reduction of inflammation (Saarela et al. 2000). The benefits have been mostly focused on the antipathogenic activity of the bacteria, indicating that enhancing antimicrobial abilities tends to be central to probiotics research. Pediocin, a cationic peptide, is known as a strong antimicrobial peptide that is produced in *Pediococcus* species (Rodriguez et al. 2002). It is strongly active against *L. monocytogenes* (Yousef et al. 1991) and induces cell autolysis by forming a protein complex on cytoplasmic membranes(Montville and Chen 1998). The antimicrobial ability of pediocin has mostly been reported in Gram-positive pathogens, while pediocin can also inflict sublethal injuries in Gram-negative bacteria (Montville and Chen 1998). In this study, PIN treatment to PA markedly enhanced the production of pediocin. Consistent with this result, PA/PINs showed higher gene expression levels of pediocin than other groups, especially *pedA* and *pedD*. Form these results, it can be hypothesized that internalization of PINs could affect the production of pediocin via expression of the *pedA* and *pedD* gene, and

its production has been reported to represent as a quorum-sensing phenomenon (Kleerebezem et al. 1997).

Notably, probiotics produce bacteriocin as the first defense system (Cleveland et al. 2001), and therefore various factors such as cultural temperature (Kalchayanand et al. 1998), pH, and pressure (Castro et al. 2015) can affect the expression of bacteriocin by upregulating genes associated with the stress response, such as heat shock proteins (HSPs) (Bove et al. 2013). Transcriptional analysis of the genes of PA with internalized PINs revealed significantly higher expression levels of HSPs (*groEL*, *groES*, and *dnaK*) than PA alone**.** The results indicated that internalization of PINs into PA caused a mild stress to induce the bacterial defense mechanism without cell death. Therefore, internalization of PINs into PA increased the expression of pediocin biosynthetic genes, modulated cell metabolisms, and activated the defense system. However, a comprehensive mechanistic study of the internalization of PINs into PA is required to unravel the mechanisms underlying the changes in expression levels of various genes in PA with internalized PINs.

In general, lactic acid or hydrogen peroxide (H_2O_2) is closely related to the antimicrobial property of probiotics (Brudzynski 2006). Interestingly, the internalization of PINs by PA did not affect the production of lactic acid and H_2O_2. Thus, it is clear that the enhanced antimicrobial activity of PA with internalized PINs was largely regulated with pediocin. In contrast, SCFA production was lower in PA/PINs than PA alone, but there were no significant differences among

PA/PINs and PA. Transcriptional analysis revealed the different levels of gene expression in PA with internalized PINs. The expression level of genes for RNA metabolism, DNA metabolism, and cell wall and capsule increased whereas transcripts belonged to carbohydrate category decreased**.** However, finding direct relationship between the production of increased pediocin and decreased SCFAs was not possible at this time point. Further experiments are needed to verify the relationships between the internalization of PINs and the changes in cellular metabolism.

Ultimately, the study 1 can be concluded that prebiotic nanoparticles can exert tremendous effects on probiotics leading to enhanced production of antimicrobial peptides that are effective against both Gram-positive and Gram-negative pathogens. Thus, this study highlights a novel way of producing antibacterial peptide in probiotics through intracellular stimulation by internalized inulin nanoparticles as a prebiotic that holds a great promise to replace antibiotics for dairy, veterinary and human applications.

Study 2. Investigation of the effect of dextran nanoparticles on probiotics antimicrobial property

1. Introduction

Microbiota in the gut system have garnered much attention in recent years because they interact mutually with the host health. The gut microbiota is a complex of microorganisms that lives in animal gastrointestinal tracts (Sommer and Backhed 2013). Microorganisms are known to live more than100 trillion in the gastrointestinal tract and these organisms plays important roles in biological processes, such as nutrient utilization, resistance against infection, and host metabolism (Wilson 2019). Depending upon their composition and communication, they can influence host physiology, including obesity, type 2 diabetes, cardiovascular disease, cancer, and brain development and antimicrobial activity (Corfield 2018; Gerritsen et al. 2011; Schwiertz 2016; Szablewski 2018; von Martels et al. 2017; Waldman and Balskus 2018). Because of advanced high-throughput sequencing technologies, it is now clear that diet has a considerable effect on changing the composition of the gut microbiota (Lee et al. 2017). Therefore, probiotic studies are now also focusing on the alterations of the host gut microbiome after probiotic administration (Marchesi et al. 2016). Probiotics can manage the gut microbiota by increasing the number of beneficial microorganisms and by suppressing the number of

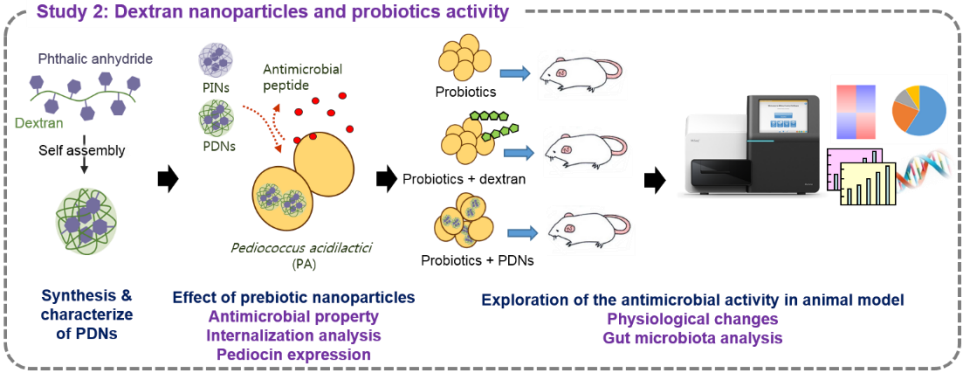
harmful microorganisms, which allows them to manage gut infections (Round and Mazmanian 2009). Therefore, probiotics can confer health benefits on the host when it is administered in adequate amounts (Hill et al. 2014).

Enteric pathogens are a major cause of infections in the gastrointestinal track worldwide. 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 feed the use of antibiotics are prohibited. Therefore, finding efficient alternatives to antibiotics is a global issue (Czaplewski et al. 2016). Since probiotics are generally considered safe and can confer health benefits to the host when adequate amounts are administered, interest in the use of probiotics as replacement of antibiotics has been growing (Fuller 1989; Wan et al. 2018). Lactic acid bacteria are commonly used as probiotics for alternatives of antibiotics. Among lactic acid bacteria, such as *Lactobacillus* spp. and *Pediococcus* spp. are used widely since these probiotics have antimicrobial properties in many pathogens (Cotter et al. 2013; Di Giancamillo et al. 2008; Mountzouris et al. 2007). These probiotics produce antimicrobial molecules (e.g., lactic acid and bacteriocins) that enable them to inhibit the colonization of pathogens, modulate the immune system, and enhance nutrient absorption (Oelschlaeger 2010; Rastall et al. 2005). A number of strategies, including biological, physical, and chemical methods, have been employed to enhance the biological activity of probiotics (Arokiyamary and Sivakumaar 2011; Kaur et al. 2013).

To induce the growth or activity of probiotics, among many strategies use of prebiotics are one them. Prebiotics are generally defined as indigestible food ingredients that induce the growth or activity of beneficial microorganisms in the gastrointestinal tract and provide favorable health effects to the host. Dextran consists α(1→6) glycosidic linkages between straight chain glucose molecules and α(1→3) linkages between branches; these linkages cannot be digested by pancreatic enzymes in the upper gastrointestinal (GI) tract but can be fermented by the gut microbiota (Sarbini et al. 2014). Therefore, dextran has been increasingly used as a prebiotic source due to its complex branched glucans (Olano-Martin et al. 2000; Rastall and Gibson 2015; Sarbini et al. 2014). Based on these information of prebiotics, many strategies have been employed using prebiotics to enhance the growth or activity of beneficial probiotics (Gibson and Roberfroid 1995; Gourbeyre et al. 2011). In study 1, phthalyl inulin nanoparticles (PINs) was synthesized as prebiotics and demonstrated that PINs were able to be internalized by *Pediococcus acidilactici* (PA) (Kim et al. 2018). This internalization increased the expression of pediocin biosynthetic genes, modulated cell metabolism, and activated the PA defense system. The enhancement of pediocin expression resulted in higher antimicrobial activity against on both Gram-negative and Gram-positive pathogens, demonstrating that the internalization of prebiotic nanoparticles affected probiotics through mild intracellular stimulation, which resulted in the enhanced production of pediocin as an antimicrobial peptide that is effective against pathogens.

In study 2, to confirm the suppression of pathogenic gut infection and alterations in the gut microbiota *in vivo* using different types of prebiotic nanoparticles. A new type of phthalyl dextran nanoparticles (PDNs) was developed and used them to treat PA (Figure 30). The changes in the antimicrobial activity of PA was analyzed and explored the effects on PA in response to the internalization of PDNs *in vitro*. For further exploration, animal models was used to explore antimicrobial activity against pathogens and the changes in the gut microbiota population by the administration of PDNs. Study 2 demonstrates that the increased antimicrobial activity of probiotics induced by prebiotic nanoparticles is applicable to animal models and can alter the population of the gut microbiota *in vivo*.

A



B

Study	Category	Item
Developing a new type of phthalyl dextran nanoparticles and analyzing the changes in PA	Synthesis of phthalyl-dextran	dextran, hydrophobic group (phthalyl anhydride) HNMR, SEM, DLS, ELS
	Antimicrobial property	Co-culture assay (viable cell counts) Pathogen agar layered test (inhibition zone)
	Tracking internalization	Confocal microscope (FITC)
	Effects of PDNs on probiotics	Viable cell counts, pH and SCFA (GC) Pediocin (protein, mRNA, activity) HSP mRNA level
Exploration of the antimicrobial activity against pathogens in animal model	Experimental design	Animal (murine) Sampling (body weight, colon, feces)
	Physiological changes	Body weight, feed intake Colon length
	Gut microbiota analysis	Composition of OTUs Relative abundances of OTUs
	Specific microbial analysis	Viable cell counts, DNA (qPCR) Phylum and genus level

Figure 30. Overview of Study 2. (A) Graphic abstract and (B) research contents of study 2.

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 sorbitol, MacConkey agar and brain heart infusion (BHI) broth were purchased from BD Difco (Sparks, MD, USA).

2) Synthesis of phthalyl dextran nanoparticles (PDNs)

Phthalyl dextran nanoparticles were synthesized and characterized according to a previously described method(Kim et al. 2018). Briefly, dextran (1 g, MW = 4000 g mol^{-1}) was added to 5 ml of dimethyl formamide, and then, 0.2 ml of 5 % sodium acetate (w/v) was added as a catalyst for the reaction. Subsequently, phthalic anhydride was added to the dextran solution at a 2:1 molar ratio. The reaction was performed at 40 °C for 24 h with nitrogen, and then, the PDNs were lyophilized and stored at -20 °C until use. For the characterization of PDNs, the contents of the phthalyl group in the PDNs were confirmed by 600 MHz ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (AVANCE 600, Bruker, Germany). The surface topography of the PDNs was analyzed using a field-emission scanning electron microscope (FE-SEM) with SUPRA 55VP-SEM (Carl Zeiss, Oberkochen,

Germany). 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).

3) Bacterial cultures

All bacterial strains were cultured in their corresponding medium: *Pediococcus acidilactici* (PA) in MRS broth, Gram-negative *Salmonella* Gallinarum, ETEC K88, and EHEC O157:H7 in LB broth, and Gram-positive *L. monocytogenes* in BHI broth. Gram-positive and Gram-negative 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. Pathogenic strains were provided by the Korean Agricultural Culture Collection (KACC, South Korea).

4) Antimicrobial assay by co-culture and agar diffusion test

The antimicrobial activity of PA against pathogens was determined using co-culture assays and agar diffusion tests. For co-culture assays, 1.0 x 10^6 CFU/ml of the indicated Gram-negative pathogens were co-cultured with 1.0 x 10^6 CFU/ml of PA [treated with or without 0.5 % (w/v) PDNs or dextran] in MRS broth for 8

h at 37 ℃ in aerobic conditions in a shaking incubator (255 rpm). Co-culture samples were then spread onto MacConkey agar. For agar diffusion tests, 120 μl of the indicated Gram-negative pathogens (2.0 x 10^8 CFU/ml) were spread onto an LB agar plate. Next, a paper disc was placed on the agar spread with pathogen and 120 μl (2.0 x 10^8 CFU/ml) of PA [cultured with 0.5 % (w/v) PDN or dextran] was dropped onto the paper disc. The zone of inhibition was measured after 20 h of incubation at 37 ℃. For Gram-positive pathogens, 1.0 x 10^5 CFU/ml of PA and *L. monocytogenes* were indicated with BHI broth and spread onto Oxford agar for co-culture assays. For agar diffusion tests BHI agar was used.

5) Tracking the internalization of dextran and PDNs by probiotics

The internalization of dextran and PDNs was measured as previously described (Kim et al. 2018). Initially, fluorescence isothiocyanate (FITC)-labeled PDNs and dextran were prepared. To observe the time-dependent internalization of dextran and PDNs by probiotics, *Pediococcus acidilactici* 175 (PA, KCTC 21088) (2.0 x 10^5 CFU/ml) were inoculated into 1 ml of MRS broth, treated with 0.5 % (w/v) FITC-dextran or FITC-PDNs and incubated for the specified time. For temperature-dependent internalization, PA was incubated with 0.5 % FITC-dextran or FITC-PDNs for 2 h at 4, 25 and 37 ℃. To observe the transporter-dependent internalization of nanoparticles by probiotics, glucose, galactose and fructose were used as blocking agents. After PA was pre-incubated with 10 % (w/v)

glucose, galactose or fructose for 10 min at 37 °C, 0.5 % FITC-dextran or FITC-PDNs were added for 2 h at 37 °C. After each experiment, the samples were washed with PBS and analyzed by flow cytometry and confocal laser microscopy (SP8 X STED, Leica, Wetzlar, Germany).

6) Analysis of pediocin production

PA was treated with or without 0.5 % (w/v) PDNs or dextran. Pediocin production was determined using Bradford assays, pediocin activity assays and RT-PCR. All experiments were performed as described previously(Kim et al. 2018). The Bradford assay was used to determine the protein concentration of purified pediocin. The pediocin was purified using the ASM purification method and was quantified using a standard curve of bovine serum albumin. To determine the specific activity of pediocin, the diameter of the *L. monocytogenes* zone of inhibition after incubation with culture supernatants (pH 5.5) was measured and expressed as arbitrary units (AU) per ml.

7) 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 extractions were conducted according to the manufacturer's

instructions. After the isolation of RNA, cDNA was synthesized from 1 μg of RNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover purchased from TOYOBO CO., LTD (Dojima, Osaka, Japan). RT-PCR was performed with SYBR qPCR Mix using a one-step real-time PCR protocol. The primer sequences were designed as described by Fernandez *et al*.(Fernandez et al. 2014) and are listed in Table 9. For relative quantification, 0.01 ng of 16s rRNA cDNA was used with 1 ng of primer. The relative gene expression was calculated using the ΔΔCt method. The target gene expression was normalized to the relative expression of the 16s rRNA gene used as an internal control in each sample. The data are presented as the relative fold-change over the probiotic control group.

8) Animal experimental procedures and measurements

Studies were performed using five-week-old BALB/C male mice in accordance with international ethical guidelines. The Institutional Animal Care and Use Committee at Seoul National University approved the animal experiments (SNU-170531-1-1). 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 four groups (10 BALB/c mice per group). The control group continued to be fed as before. The T1 group was administered a single dose of 10^8 CFU *Pediococcus acidilactici* (PA) in saline solution via oral gavage. The

T2 and T3 groups were administered single doses of dextran (0.5 wt.-%)-treated PA or PDNs (0.5 wt.-%)-treated PA, respectively, as described above. After 7 days on the test diets, EHEC O157:H7 (10^9 CFU) was administered with 0.2 ml of 1% $NaHCO_3$ (treated before administrating EHEC O157:H7 for 30 min) to the mice via oral gavage for three days, while the test groups were continually administered the test diets for six 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, PA and *E. coli* were counted as viable CFU from daily fecal samples (10 mg/ml). Feces were spread onto both MRS agar and MacConkey sorbitol agar and incubated for 20 h at 37 °C. At the end of the experiment, mice were sacrificed by CO_2. Intestinal samples and feces were collected from the intestines. Collected feces were used for counting viable cells and for DNA extractions.

9) DNA extraction and sequencing

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 reported by Brown *et al*., and qPCR was performed as previously described(Brown et al. 2016). Primers are listed in Table 10.

For Illumina MiSeq, the V4 region of the bacterial 16S rRNA gene was amplified from the total extracted DNA using Takara Ex Taq polymerase (Takara Bio, Shiga, Japan) and the 515F-806R primer pair(Caporaso et al. 2010a). The amplification program consisted of 1 cycle at 94 °C for 3 min, followed by 40 cycles at 94 °C for 45 sec, 55 °C for 1 min, and 72 °C for 1.5 min, and finally, 1 cycle at 72 °C for 10 min. DNA libraries were constructed using the Illumina TruSeq DNA Sample prep kit and paired-end sequencing (2 × 300 bp) of the amplicons was completed on an Illumina MiSeq (Macrogen, Seoul, Republic of Korea). The 16S rRNA gene sequences identified in this study were deposited in the NCBI Sequence Read Archive (SRA) database under accession number SRR7867415.

Table 10. List of the primers used in this study

	Primer sequence (5'-3')	Size (bp)
Pediococcus acidilactici	r: GGACTTGATAACGTACCCGC f: GTTCCGTCTTGCATTTGACC	449
Intimin	r: CATTGATCAGGATTTTTCTGGTGATA f: CTCATGCGGAAATAGCCGTTA	102
Bifidobacterium spp	r: CTCCTGGAAACGGGTGG f: GGTGTTCTTCCCGATATCTACA	549–563

10) Fecal microbiota analysis

Raw sequence reads were quality trimmed using FastQC v0.11.5 and FASTX-Toolkit v0.0.13 software. Trimmed paired-end reads were merged using FLASH v1.2.11 (parameter settings: -m 50 -M 205)(Magoc and Salzberg 2011) and demultiplexed. The microbial communities were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 software(Caporaso et al. 2010b). The reads were clustered into operational taxonomic units (OTUs) by subsampled open-reference OTU picking at 97 % identity with the GreenGenes 13-8 database as described previously(DeSantis et al. 2006). OTU picking was completed using the usearch61 method (parameter setting: -s 0.1)(Edgar 2010). The representative sequences were aligned using PyNAST(Caporaso et al. 2010a). The representative sequences were taxonomically assigned using the uclust consensus taxonomy assigner. The OTU tables were normalized to 16,660 reads per sample by single rarefaction and further analyzed.

The alpha diversity index (observed OTUs) was calculated from 16,660 sequence reads per sample through rarefaction with 10 iterations. Principal coordinate analysis (PCoA) was performed based on weighted UniFrac distances, and the effect of treatment on the microbial community was evaluated using ANOSIM statistical tests using the compare_category.py script in QIIME with 999 permutations. The abundance of microbial taxa was expressed as a percentage of the total number of 16S rRNA gene sequences.

11) Statistical analysis

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

3. Results

1) Synthesis and characterization of PDNs

The reaction scheme for phthalyl dextran (PD) is shown in Figure 31. To prepare the PDNs, hydrophobic phthalic groups were introduced into dextran. The hydroxyl groups in dextran and the carboxylic acids in phthalic acid were reacted through an ester bond. After the reaction, the degree of the phthalic groups in the PD was confirmed by ^{1}H-NMR measurement (Figure 31C**)**. The protons of dextran and phthalic acid in PD appeared at 3.8 and 7.4-7.7 ppm, respectively. The phthalic groups in PD were 27.6 mol-%, based on the integration of protons in both the dextran and phthalic groups. The morphologies of the PDNs, as observed by SEM, were spherical with sizes on the nanometer scale (Figure 31B). Moreover, the size and zeta potential of the PDNs were measured by DLS and ELS (Figure 31D). The average size of the PDNs was 238.7 nm with a polydispersity index of 0.220. The zeta potential was -27.32 mV due to the nonreacted carboxylic acids in the phthalic group of the PDNs.

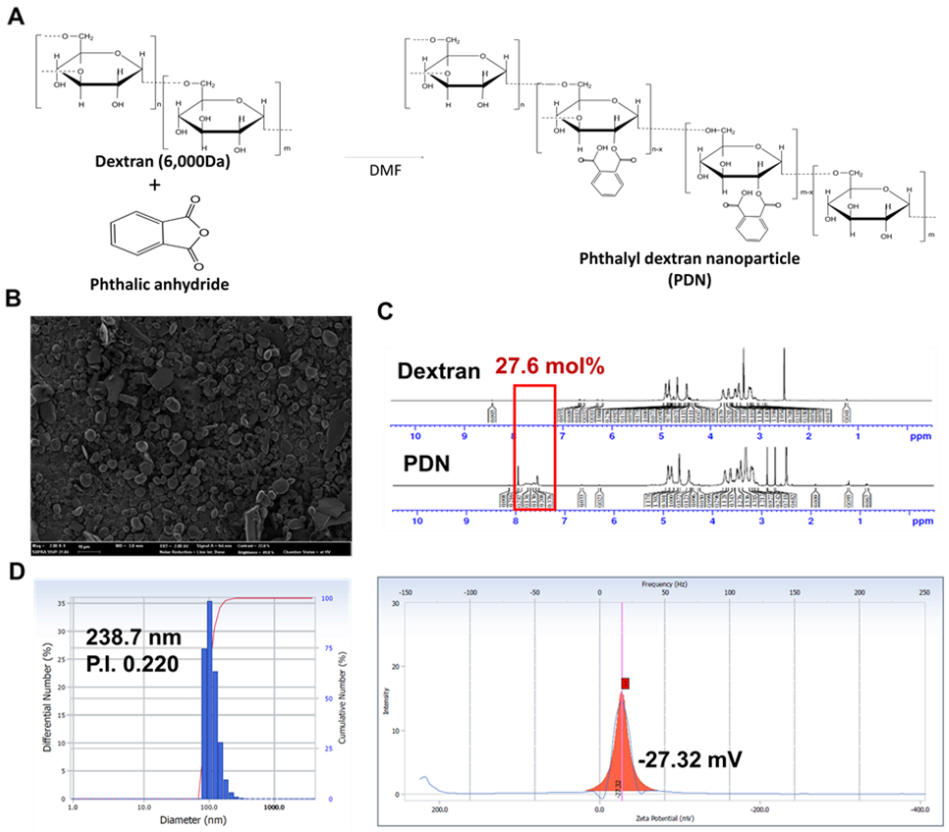


Figure 31. Characterization of PDNs. (A) Chemical reaction scheme for the synthesis of PDNs. For the reaction in each PDNs synthesis, the molar ratio between phthalic anhydride and dextran was as follows: 2:1. (B) Morphology of PDNs observed by SEM. (C) Calculation of mol.-% of phthalic acid in PDNs by 1H-NMR spectroscopy. (D) Measurement of the sizes of PDN by DLS and zeta-potential by ELS. Magnification: 10,000X; Scale bar=2 μm.

2) Antibacterial activity of PA after treated with PDNs

To evaluate whether the antimicrobial potency of PA was changed by the treatment of PDNs, PA treated with PDNs (PA/PDNs) was tested with PA internalized with PINs (PA/PINs) by co-culture assay against the Gram-negative pathogens, *Salmonella* Gallinarum, EHEC O157:H7 and the Gram-positive pathogen *L. monocytogenes* (Figure 32). Both PA/PINs and PA/PDNs showed higher antimicrobial activity than PA or PA treated with inulin or dextran. However, there was no difference on antimicrobial activity of among PA/PINs and PA/PDNs. Therefore, PDNs was used for further experiment to demonstrate if the previous mechanism is common with different type of prebiotic nanoparticles.

To evaluate more advanced antimicrobial activity of PA/PDNs, Gram-negative pathogens, *Salmonella* Gallinarum, ETEC K88, EHEC O157:H7 and the Gram-positive pathogen *L. monocytogenes* were used **(**Figure 33A-D). In co-culture assays, PA/PDNs showed significantly higher antimicrobial activity against both Gram-negative and Gram-positive pathogens than untreated PA or PA treated with dextran alone (PA/D). In addition, agar diffusion tests was conducted to see the direct effects of the antimicrobial peptides produced by PA. The agar diffusion test results were similar to the co-culture assays; PA/PDNs showed wider zones of inhibition than PA/D or PA alone. These results suggested that the internalization of PDNs by PA induced their antimicrobial properties and affected their production of antimicrobial peptides.

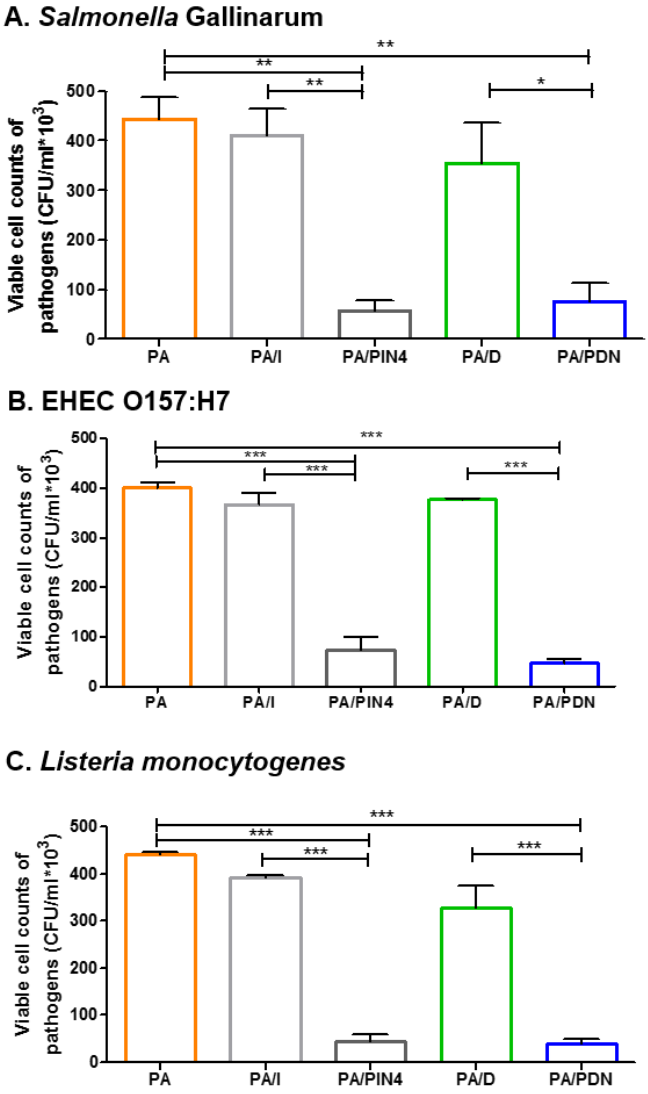


Figure 32. Antimicrobial efficacy of PINs and PDNs-treated probiotics against pathogens. (A-C) PA treated with PINs or inulin were cultured with Gram-negative *Salmonella* Gallinarum, EHEC O157:H7 or Gram-positive *L. monocytogenes* and the growth inhibition was calculated by CFU.

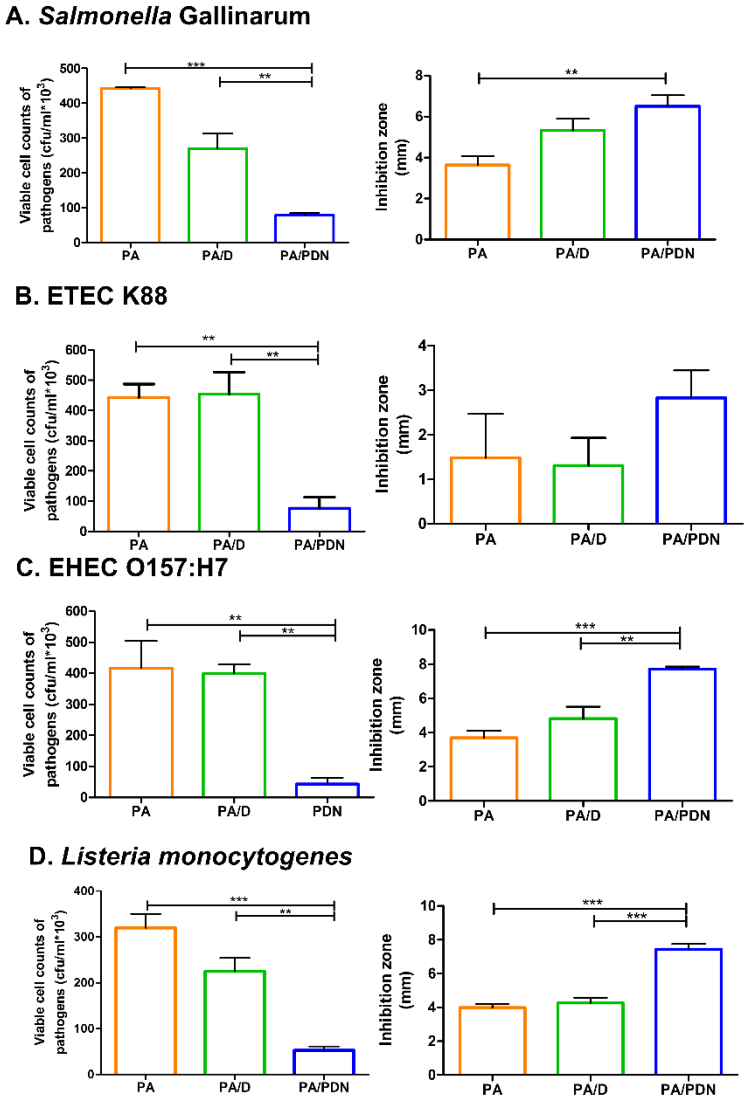


Figure 33. Antimicrobial efficacy of PDNs-treated probiotics against pathogens. (A-C) PA treated with PDNs or dextran was cultured with Gram-negative *Salmonella* Gallinarum, ETEC K88, and EHEC O157:H7, (D) Gram-positive *L .monocytogenes* and the growth inhibition was calculated by CFU and the diameters of the zones of inhibition.

3) Internalization of PDNs by probiotics

To investigate the internalization of PDNs by *Pediococcus acidilactici* (PA), fluorescence isothiocyanate (FITC) was conjugated to the PDNs. The internalization of the PDNs was confirmed by CLSM and FACS (Figure 34). Initially, time-dependent internalization was measured. The PDNs were able to be internalized by PA within 3 min and that their internalization increased with time **(**Figure 34**),** although FITC-conjugated dextran alone was able to enter PA by diffusion within 3 min of incubation **(**Figure 35**)**. Next, the temperature-dependent internalization of PDNs by PA was determined. The results indicated that the internalization of PDNs was higher after incubation for 2 h at 37 °C than after incubation at 25 °C or 4 °C, indicating that the internalization of PDNs increased with an increase in the incubation temperature, suggesting an energy-dependent mechanism.

To examine the mechanism of transporter-mediated internalization, PA was pre-incubated with 10 % (w/v) glucose, fructose or galactose, followed by treatment with 0.5 % (w/v) FITC-PDNs for 2 h. Pretreatment of PA with glucose impeded internalization by approximately 74.6 %, whereas pretreatment of PA with galactose and fructose impeded internalization by 55.1 % and 46.8 %, respectively **(**Figure 34**)**. However, there were no significant differences in the internalization of dextran by diffusion after PA was pretreated with sugars, suggesting that glucose transporters play a dominant role in the internalization of PDNs, which is similar to what was observed with PINs (Kim et al. 2018).

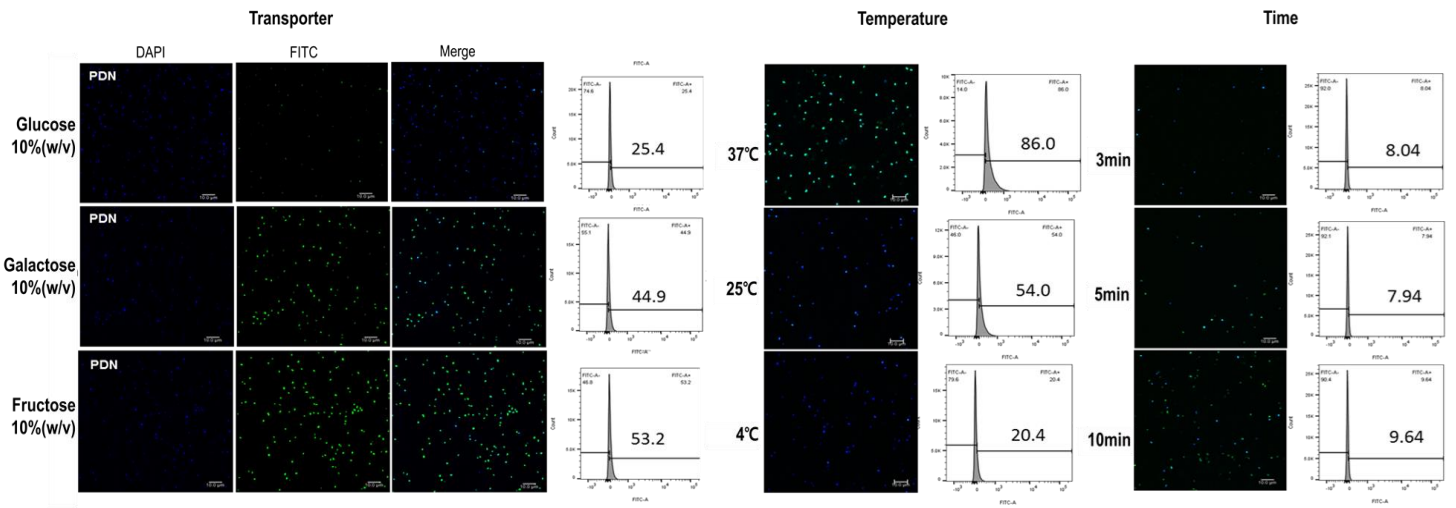


Figure 34. Analysis of the internalization of PDNs by PA. The internalization of PDNs by PA in a various conditions was analyzed by confocal microscopy and FACS. For transporter-, PA pre-incubated with 10 % (w/v) sugars, was treated with 0.5 % (w/v) FITC-PDNs for 2 h at 37 °C. For temperature-, PA was treated with 0.5 % (w/v) FITC-PDNs at different temperatures for 2 h. For time-, PA was treated with 0.5 % (w/v) FITC-PDNs for 3, 5, and 10 min. FITC-PDNs is shown in green, and PA is stained

blue with DAPI. Confocal images and FACS analysis were performed after the treatments. Confocal images and FACS data shown are representative of three independent experiments. Scale bar = 10 µm.

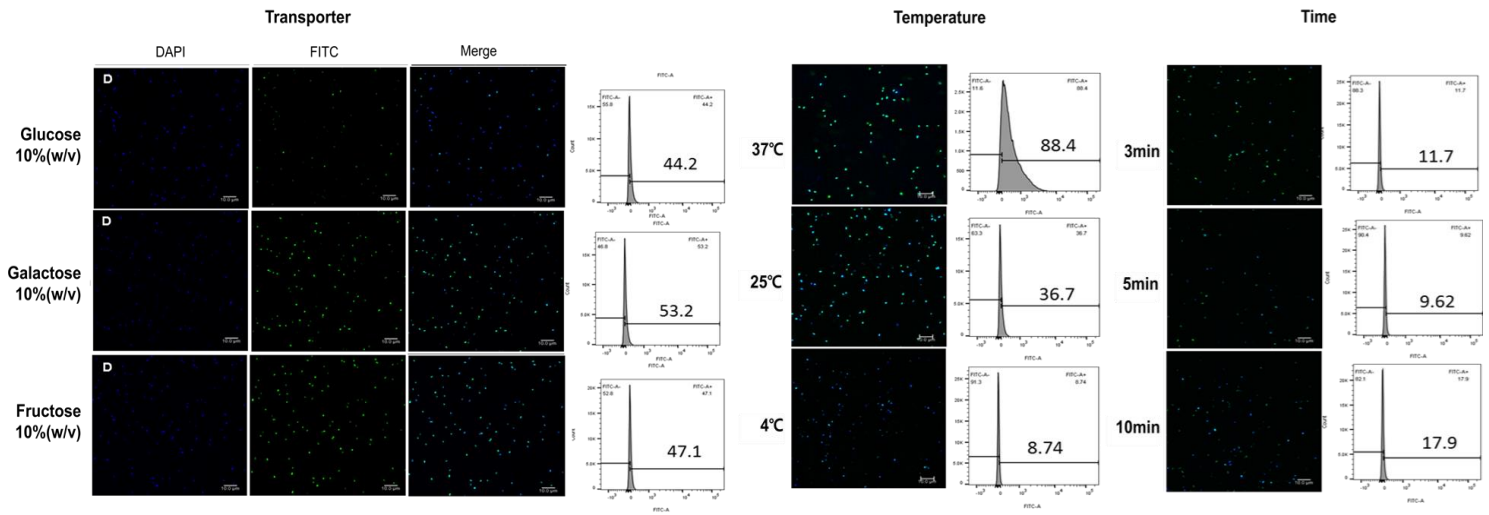


Figure 35. Analysis of the internalization of dextran by PA. FITC-dextran is shown in green, and PA is stained blue with DAPI. Confocal images and FACS analysis were performed after the treatments. Confocal images and FACS data shown are representative of three independent experiments. Scale bar = 10 µm.

4) Effects of PDN internalization on PA pediocin production and stress response

To further determine the effects of PDN internalization by PA on the induction of antimicrobial activity, changes in growth was measured, pH and short chain fatty acid (SCFA) production. There were no remarkable differences among the treatment groups (Figure 36A-C). The production of the antimicrobial peptide, pediocin, by PA was quantified using a Bradford assay, quantitative real-time PCR (RT-PCR), and pediocin activity tests. First, the amount of pediocin isolated from untreated PA and dextran- or PDNs-treated PA was quantified using a Bradford assay (Figure 36D). The production of pediocin by PA/PDNs was 2.2-fold and 1.7-fold higher than PA and PA/D, respectively. The specific activity of pediocin (represented as arbitrary units) was measured (Figure 36E). The results showed that PA/PDN had 7.0-fold and 5.8-fold higher pediocin activity than PA and PA/D, respectively.

To validate the pediocin production by PA at the genetic level, RT-PCR was used to measure the expression of genes involved in pediocin biosynthesis (Figure 36F). The relative expression levels of all four pediocin biosynthesis genes were higher in PA/PDNs than in PA or PA/D. In particular, the expression of the *pedA* gene was significantly higher (3.3-fold) in PA/PDNs than in untreated PA. These results supported the hypothesis that the internalization of PDNs by PA enhanced the antimicrobial ability of PA through the induction of pediocin production.

Based on previous results indicating that the internalization of nanoparticles induced the stress response of PA, the expression levels of several heat shock-related genes was measured, including *groEL, groES, dnaK* and *dnaJ*, after the internalization of PDNs by PA **(**Figure 37**)**. The expression levels of *groEL, groES,* and *dnaK* in PA/PDNs were higher than in PA or PA/D. Notably, the expression of *groEL* in PA/PDNs was significantly higher (3.2-fold) than in PA, suggesting that the internalization of PDNs by PA induced the stress response of PA.

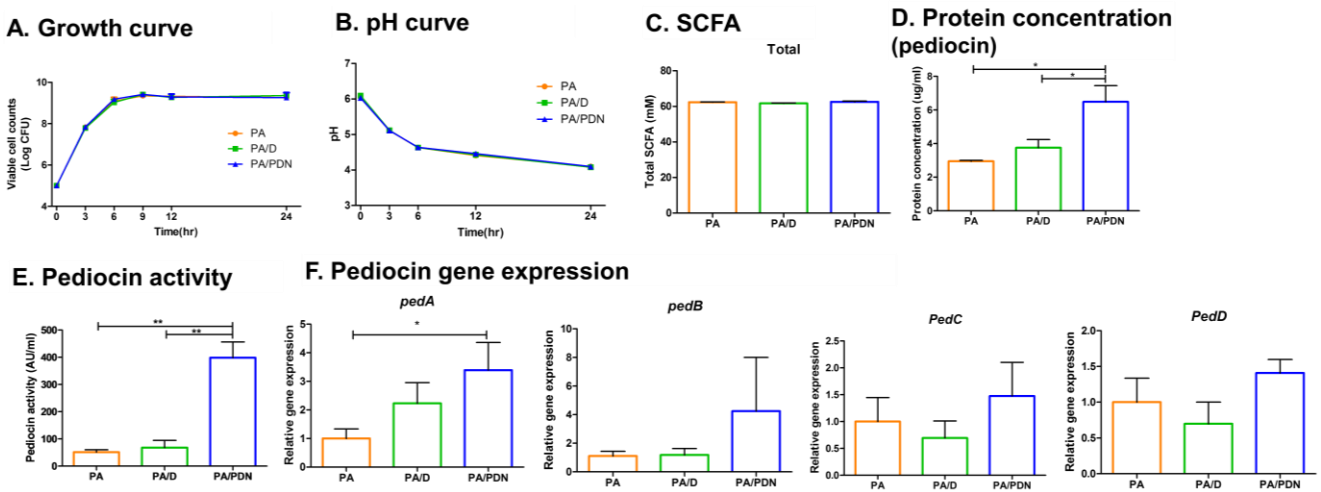


Figure 36. Analysis of biological effect of PDNs in probiotics. (A) Measurement of growth, (B) pH and (C) SCFA content of PA. (D) Quantification of pediocin production by the Bradford assay. (E) Determination of the pediocin activity (AU/ml) (F) Relative mRNA expression of *pedA-D* compared with 16S rRNA expression.

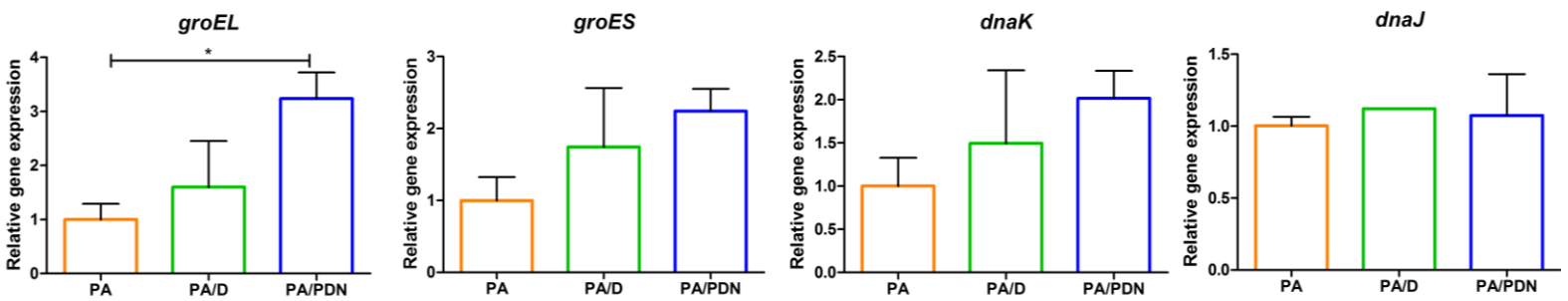


Figure 37. Analysis of gene expression related to stress response in PDNs-treated probiotics. The expression levels of *groEL*, *groES*, *dnaK* and *dnaJ* relative to 16S rRNA were quantified by RT-PCR

5) Physiological changes in mice

In study 2, mouse feeding experiments was performed, to evaluate the antimicrobial effects of PA with internalized PDNs in animals. Mice were fed a basal diet supplemented with PA (T1, PA 10^8 CFU/mouse), PA treated with dextran [T2, PA 10^8 CFU + 0.5 % (w/v) dextran/mouse], PA treated with PDNs [T3, PA 10^8 CFU + 0.5 % (w/v) PDNs/mouse], or a control unsupplemented diet (C, without probiotic bacteria) for 7 days. After 7 days, one pathogen, EHEC O157:H7, was chosen to test the antimicrobial effect of PA with internalized PDNs in animals. EHEC O157:H7 was orally administered to all test groups for 3 days. At the same time, test diets were continually administered for a total of six days. The growth rates and food intake of all mice were measured over the course of the entire trial (data not shown). Interestingly, groups that were fed diets supplemented with probiotics had increased body weight after 13 days, compared to their body weights on day 7 (Figure 38A). In particular, the body weights were increased for the T2 and T3 groups, while the body weights were decreased for the control group on day 13 compared to day 7. Moreover, the average food intake per animal for the T3 group was higher than other groups (Figure 38B).

Changes in colon length following the trial were also measured. The T3 group had the longest colon length compared to the other groups (Figure 38C), although the increase in the colon length was positively correlated with the increase in body weight (correlation ratio: 0.408). Overall, the results indicated that mice fed

probiotics, probiotics with prebiotics, or PDN-internalized probiotics had reduced negative effects of pathogenic gut infection.

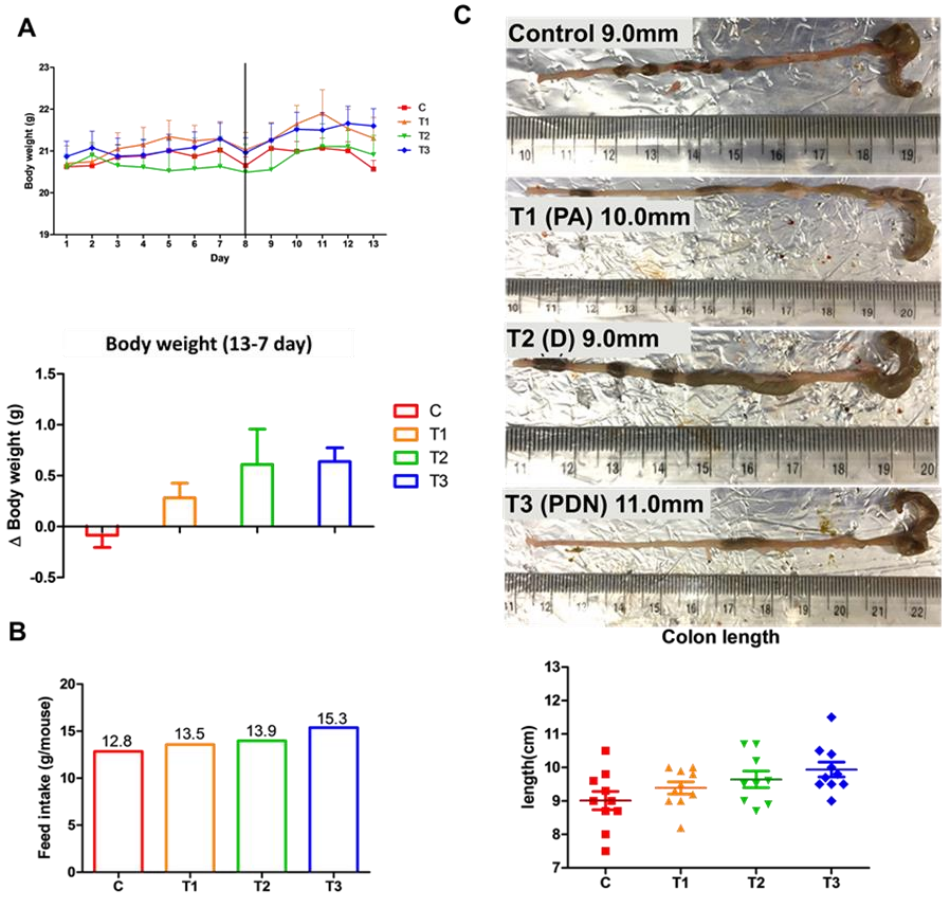


Figure 38. Physiological effects on mice. (A) Change in body weight measured at day 8 and 13. (B) The mean of feed intake of mice over a period of 6 days post to the infection with EHEC O157:H7. (C) Length of colon after the trial.

6) Microbial analysis in a murine model

Viable cell count, real-time PCR (qPCR), and phylum-level identification of the taxonomic units were 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 sorbitol agar and MRS agar daily, beginning on the first day of *E. coli* treatment (Figure 39, Figure 40). At the start of oral *E. coli* treatment, the intestinal tracks of all four mouse groups were not colonized by EHEC O157:H7 (Figure 39A). After one day, the mean *E. coli* count was approximately 4 $\log_{10}$ (CFU/mg of feces) in the intestinal tracks of all groups. On days 1 through 5 after oral administration of EHEC O157:H7, the average fecal *E. coli* contents were not significantly changed in the control group. The viable bacterial counts in the control group were approximately 3.98 to 3.69 $\log_{10}$ throughout the experiment. However, on day 2, the viable number of EHEC O157:H7 cells dramatically decreased from 3.85 to 2.98 $\log_{10}$ in the T3 group, while the T1 and T2 groups showed no differences in EHEC O157:H7 counts. At the end of the experiment (day 5), the mean number of viable EHEC O157:H7 cells was 3.69 $\log_{10}$ for the control group, 3.00 $\log_{10}$ for T1, 2.80 $\log_{10}$ for T2, and 1.57 $\log_{10}$ for T3. Interestingly, the T3 group was significantly different from the control group. On the other hand, overall viable bacterial cells counted on MRS agar remained in the range of 5 to 6.5 $\log_{10}$ throughout the experiment, suggesting that the control group had a healthy gut microbiota over the course of the experiment (Figure 40A).

To support the viable cell count results and due to the limited selectiveness of MRS agar, qPCR was used. Intimin was amplified as a marker for EHEC O157:H7 (Figure 39B). Intimin levels were significantly lower for the three groups that were orally administered probiotics (T1: 1.78-fold, T2: 2.05-fold, and T3: 0.95-fold) compared to the control group (4.20-fold), indicating that probiotics eliminated pathogens from the gut. Although not very significant, the amount of *Pediococcus acidilactici* in the PA-fed groups (T1: 8.29-fold, T2: 6.20-fold and T3: 7.24-fold) was slightly higher than in the control group (6.18-fold) (Figure 40B). From fecal samples, the relative abundance of *Bifidobacterium* spp. was also analyzed by qPCR. It was found that *Bifidobacterium* spp. (Figure 40C) were higher in the T2 and T3 groups than in the control. The T3 group in particular had the lowest level of pathogen among all the groups. These results indicated that feeding mice PA/PDNs improved the antimicrobial activity of PA more than feeding them dextran itself or PA alone.

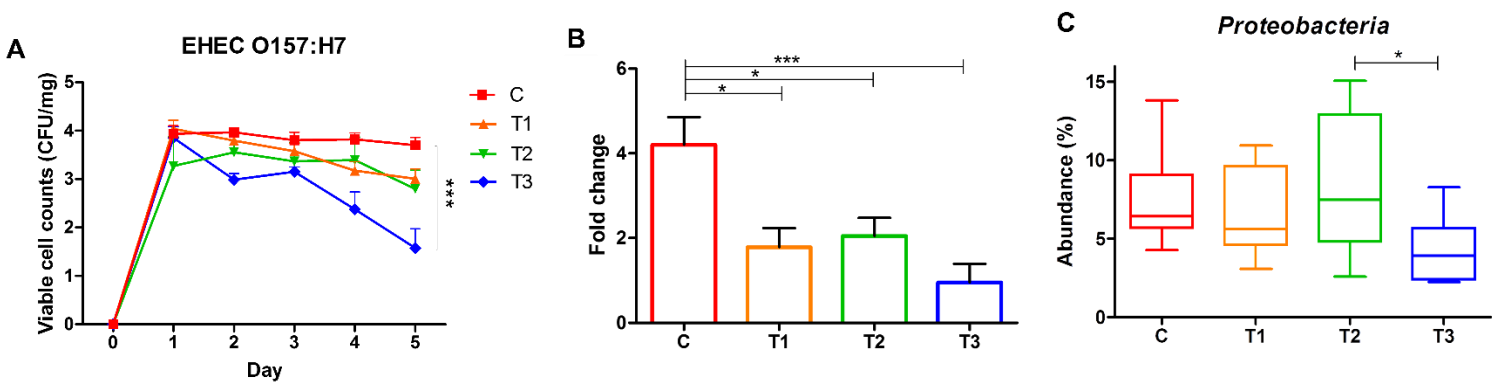


Figure 39. Number of EHEC O157:H7 cells in the intestinal microflora. (A) The viable cell counts of EHEC O157:H7 in mouse fecal samples were assayed on MacConkey sorbitol agar daily. (B) The ratio of EHEC O157:H7 (intimin) in fecal samples. (C) The proportional change in the *Proteobacteria* phylum in the intestinal microbiota.

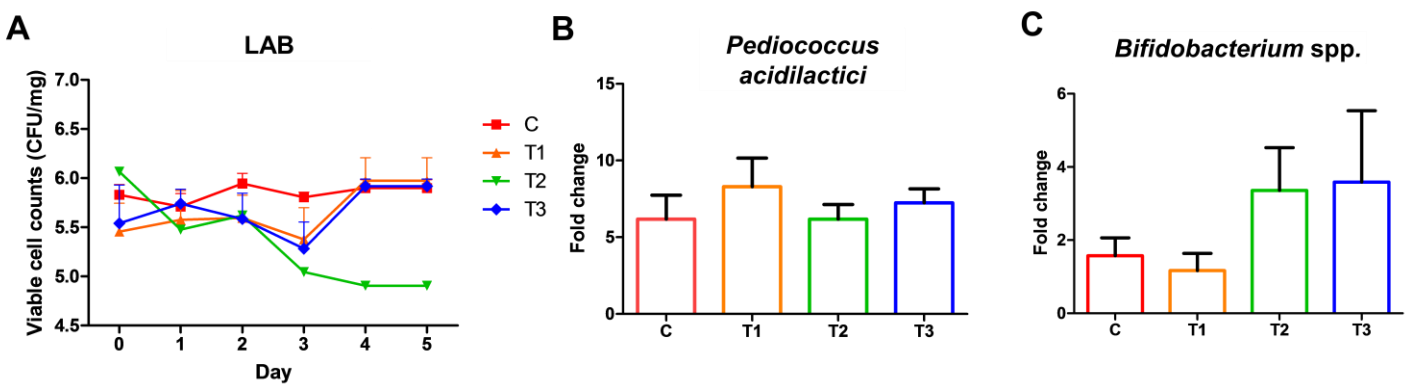


Figure 40. The number of probiotic strains in the intestinal microflora. (A) The viable cell of LAB assayed in MRS Agar by their fecal samples daily. (B) The ratio of *Pediococcus acidilactici* and (C) *Bifidobacterium* spp. in the fecal samples.

7) Alteration of the gut microbiota after treatment with test diets

From 49 samples, 16S rRNA gene sequences were obtained through Illumina MiSeq sequencing. The sequences were then clustered into operational taxonomic units (OTUs) with 97% similarity. PCoA based on unweighted UniFrac distances revealed that the fecal microbiota of the mice were altered after treatment (Figure 41A). Particularly, the control and T3 groups were distinguished separately from each other.

Moreover, the alpha diversity of the four groups after treatment was compared **(**Figure 41B). The number of observed OTUs was 1692.0 (± 171.8), 1798.0 (± 155.3), 1866.0 (± 229.5), and 1946.0 (± 163.4) for the control, T1, T2 and T3 groups, respectively. The control group in particular showed the lowest microbial diversity among the groups, which was significantly lower than the T3 group.

At the phylum level, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* composed the majority of the identified taxonomic units. Among them, the abundance of *Proteobacteria* in the T3 group was lower than in the other groups and it was significantly lower than in the T2 group, which is consistent with that qPCR and viable cell count microbial analyses (Figure 39C). Moreover, in the T3 group, the abundances of the genera *S24-7* and *Anaerostipes* were significantly higher than in other groups, especially compared to the T1 group (Figure 42A). Interestingly, the abundances of *Firmicutes* and *Bacteroidetes* in the T3 group were different from the other groups (Figure 42B).

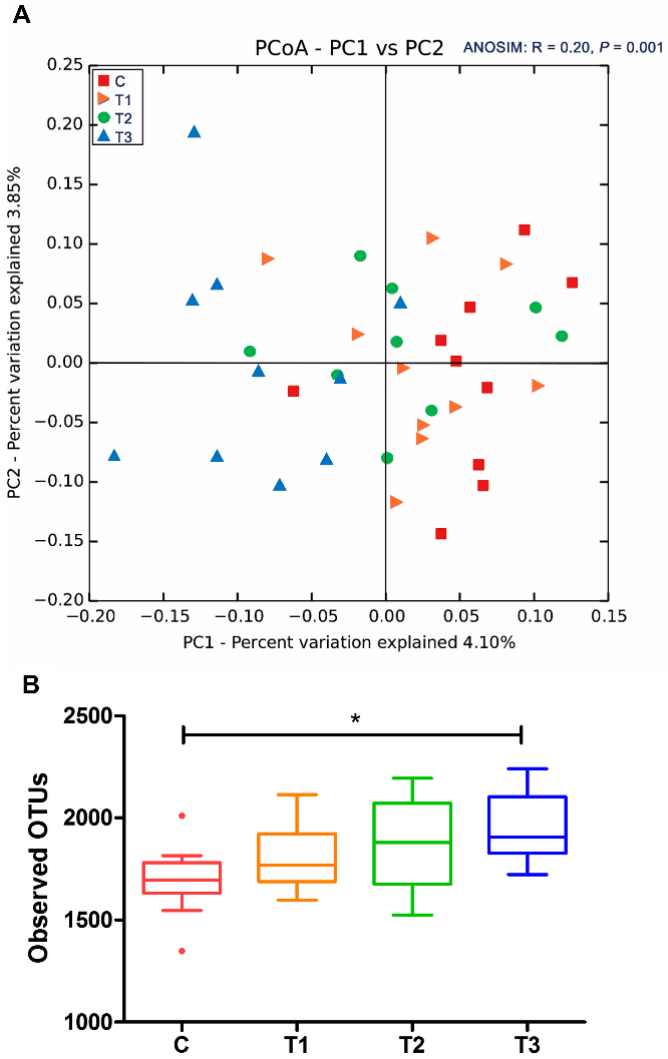


Figure 41. Shift in the intestinal microflora of mice. (A) Unweighted principal coordinate analysis (PCoA) plot of mouse intestinal microbial communities from C, T1, T2, T3 group. Subject color coding: red, control group; yellow, T1 group (PA); green, T2 group (PA with dextran); blue, T3 group (PA with PDNs). (B) The number of observed OTUs is expressed to demonstrate the richness of the groups.

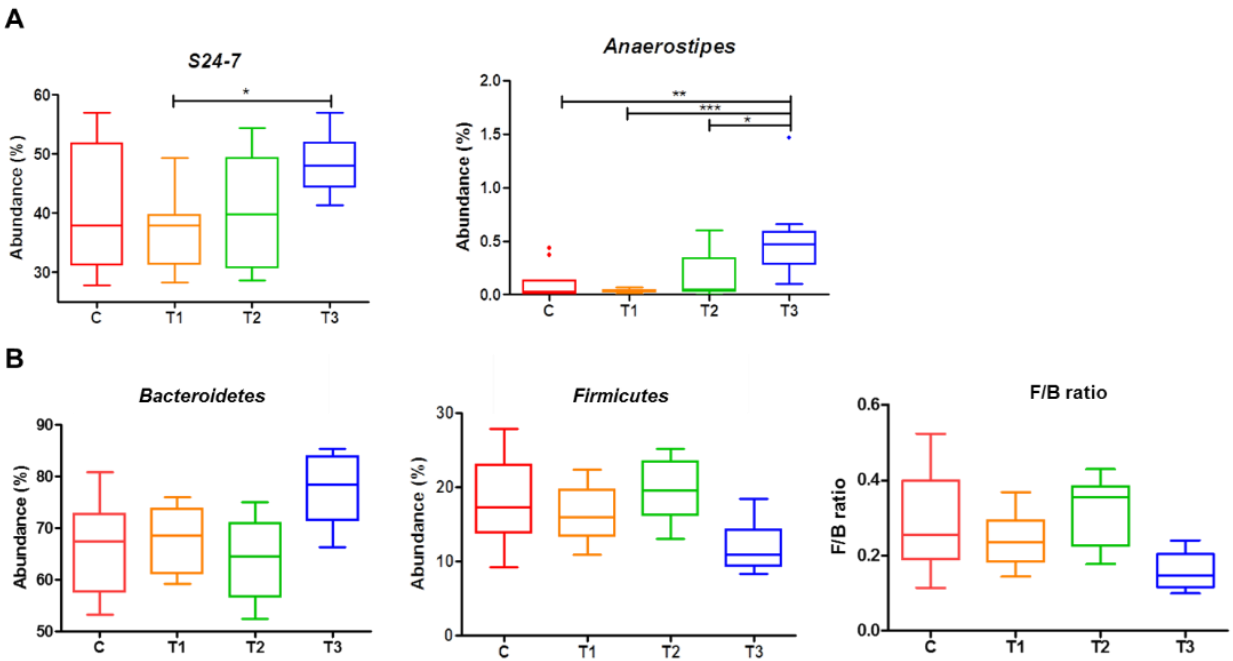


Figure 42. Bacterial abundance between groups. (A) The proportional change of genus *S24-7* and *Anaerostipes* in intestinal microbiota. (B) The abundance of *Bacteriodetes* and *Firmicutes* phylum and F/B ratio.

4. Discussion

In this study, using dextran prebiotic nanoparticles was prepared. The phthalyl anhydride was successfully conjugated with dextran through ester bonds after the reaction. Based on the integration of protons, the degree of substitution (DS) of the phthalic groups in the PDNs was 27.6 mol-%. However, increasing the DS of the phthalic group in the PDNs was difficult due to the steric hindrance of the phthalic groups. Additionally, the PDNs had a negative zeta potential due to the nonreacted carboxylic acids in the phthalic group.

Previously in study 1, it was the first to show that prebiotic nanoparticles could be internalized by probiotics in an energy-, time-, and glucose transporter-dependent manner. Similarly with the previous concept, in study 2 the internalization of PDNs by PA was also examined. Because dextran is water-soluble and can enter probiotics by passive diffusion, it was evaluated if there were differences in the internalization of dextran alone and PDNs (Barrangou et al. 2006; Saulnier et al. 2007). Accordingly, FITC was introduced to both dextran and PDNs and observed their internalization by PA in different conditions. The internalization of PDNs by PA increased with time and increased temperature, although the dextran itself was able to diffuse into probiotics similar to PDNs. To analyze if the internalization is involved with transporters, blocking assay with three type of sugars (glucose, galactose, and fructose) were used. Consistently with the internalization of PINs (phthalyl inulin nanoparticles) (Kim et al. 2018),

PDNs internalization occurred similarly. Pretreatment with glucose blocked the internalization of PDNs by PA, indicating that the nanoparticles might be recognized by a glucose transporter for their internalization. However, pretreatment with any of the three sugars tested did not impact the diffusion of dextran, indicating that dextran enters into the probiotic through passive diffusion.

In study 1, the internalization of prebiotic nanoparticles by PA enhanced the antimicrobial ability of this probiotic against pathogens through the upregulation of pediocin synthesis. To test this with PDNs, antimicrobial activity assays of PA against both Gram-negative and Gram-positive pathogens after treatment with or without dextran and PDNs was performed. Moreover, mild stress was induced in PA by the internalization of PDNs, which brought out a higher production of pediocin. This higher production of pediocin led to an increase in the antimicrobial activity of PA against pathogens, which were consistent with the previous findings (Kim et al. 2018).

The major problem during the neonatal and weaning periods is due to the acute diarrhea caused by many pathogenic *Escherichia coli* (*E. coli*). Among pathogenic *E. coli*, EHEC O157:H7 is one of the Shiga toxin-producing pathogen that causes serious disease and death. Infection with this type of pathogenic bacteria leads to hemorrhagic diarrhea, kidney failure and/or hemolytic uremic syndrome, which is very critical to younger children (Lin et al. 2017; Pennington 2010). EHEC O157:H7 produces toxin and also adheres to colonize to the host epithelial cells in the intestine using surface proteins, such as outer membrane proteins (e.g.,

intimin) (Sharma and Dean-Nystrom 2003). Therefore, strategies to prevent the colonization of pathogenic bacteria and to limit infection by inhibiting these pathogens in the intestine are needed. To protect these gastrointestinal pathogens, such as EHEC O157:H7, in animals lactic acid bacteria (LAB) are already known and used as alternative of antibiotics, because they produce antimicrobials or modulates the immune responses (Gill et al. 2000; Lin et al. 2017; Shu and Gill 2001; Shu and Gill 2002). Therefore, to validate the antimicrobial effect of PA with the treatment of PDNs and to determine whether more production of pediocin could suppress the infection of pathogenic bacteria, mouse feeding experiments with EHEC O157:H7 was performed with PA treated with or without dextran or PDNs.

To mimic the animal intestinal microenvironment the murine model was used, since mice have a normal intestinal microflora. To reduce the pathogenic infection, PA treated with or without dextran or PDNs were prefed before the administration of pathogen was because the domestic microflora plays an important role in inhibiting pathogenic infection (Shu and Gill 2002). Overall results shows that oral supplementation of PDNs-treated PA (the T3 group) protected pathogens and reduced infection by EHEC O157:H7 in mice. Although the physiological differences among the test groups were not significant, feeding probiotics with prebiotics or prebiotic nanoparticles seem to alleviate the infection. The T3 group had the highest increases in body weight and food intake. Moreover, the body weights and food intake of the T1 (PA) and T2 (PA with dextran) groups were also

higher than the control groups. It is known that when EHEC O157:H7 is infected in intestinal tract it induces toxin and inflammation of the intestine and reduces the length of colon. However, in this study mouse colon lengths were increased in all of the probiotic treatment groups compared to the control groups. Despite their was positive correlation between colon length and increase in body weight.

The composition and diversity of the gut microbiota was analyzed using sequencing methodology, because pathogenic *E. coli* triggers inflammation and disrupts the normal intestinal microflora. The microbial composition of the T3 group was found to be separated from the control group, while the T1 and T2 groups were more similar to the control group. It is known that diet may alter the composition of the gut microbiota and this separation may also came from the treatment of different diet supplements between groups (Gibson and Roberfroid 1995; Maslowski and Mackay 2011). Moreover, to analyze the diversity of gut microbiota, the number of OTUs was observed. Many researchers define higher diversity in the gut microbiota to be representative of a healthier intestinal condition (Backhed et al. 2012; Claesson et al. 2012; Jacouton et al. 2017). The number of observed OTUs was increased following the treatments of PA, PA with dextran, or PA with PDNs, compared to the control group. T3 group had the highest number of the observed OTUs among other groups, indicating that higher antimicrobial activity induced by the administration of PDNs may prevent reductions in the diversity of the microflora. Therefore, these results indicate that greater body weight, colon length, and number of OTUs can predict that PA treated

with PDNs will regulate the intestinal condition by alleviating pathogenic infection.

For the main goal to see if the increased antimicrobial property *in vitro* is maintained in *in vivo*, antimicrobial properties of PA treated with PDNs was analyzed by counting the number of the pathogenic bacteria, EHEC O157:H7. By counting the viable cells of EHEC O157:H7 and counting the intimin expression levels by qPCR of fecal samples, highest antimicrobial activity was shown in the T3 group (PA treated with PDNs). The results were consistent with *in vitro* results, which the production of pediocin and increased antimicrobial activity against Gram-negative and Gram-positive bacteria was occurred from the internalization of PDNs by PA. Therefore, the enhanced antimicrobial activity of PA/PDNs may have contributed to the decrease in pathogen load in mice, in particular, EHEC O157:H7.

The *Proteobacteria* phylum was decreased in the fecal samples from the T3 group. Species that are known to cause various diseases including *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other notable bacteria are members of the *Proteobacteria* (Mukhopadhya et al. 2012; Shin et al. 2015). Since EHEC O157:H7 belongs to the *Proteobacteria*, the decrease in this phylum can be indicated as the antimicrobial property of the treatment groups. These results can suggest that the increased production of pediocin may have excluded pathogens from the intestines of mice. However, at this time point it was unable to directly measure the amount of pediocin in the mouse intestines. Further studies are

needed to verify the direct relationship between the amount of pediocin and the decrease in pathogens in the intestine.

The effect on other bacterial species in addition to the pathogenic bacteria was also analyzed. Viable cell counts and qPCR was used to analyze the levels of LAB and *Pediococcus acidilactici*. The results demonstrated no significant decreases in *Pediococcus acidilactici* or LAB was occurred after the trial, although the ratio of *Pediococcus acidilactici* was slightly higher in the treatment groups (T1, T2 and T3) compared to that of the control group. Interestingly, *Bifidobacterium* spp. were increased in the prebiotic treatment groups (T2 and T3 groups). The results were consistent with previous results showing that treatment with dextran as a prebiotic increased the levels of *Bifidobacterium* spp. in human fecal samples (Olano-Martin et al. 2000; Sarbini et al. 2014). Based on the influence of prebiotics on the growth of *Bifidobacterium* spp., this result imply that PDNs treatment might also work as a prebiotic in the intestine.

Few individual genera were affected by the treatment of PA with or without dextran or PDNs. At the genus level, *S24-7* and *Anaerostipes* were significantly increased and the phylum *Firmicutes*-to-*Bacteroidetes* ratio (FB ratio) was reversed in T3 group compared to control group. There is not much information on the genera *S24-7* or *Anaerostipes*, however, these bacteria can be considered as an indicators of prebiotic supplementation and provide a beneficial effect to the host intestine. Schroeder *et al*. reported that after supplementation with prebiotics, the *S24-7* was highly prevalent in the mouse intestinal track (Schroeder et al.

2018). As for *Anaerostipes*, this genus is known to produce butyrate (Schwiertz et al. 2002). Butyrate is a nutrient for intestinal cells and this modulates gut integration and enhances the immune system to prevent pathogens (Canani et al. 2011; Russo et al. 2012). Therefore, these results indicate that PDNs may also work as a prebiotic in mouse intestines, although the FB ratio was altered in the T3 group and future investigations should also consider if prebiotic nanoparticles themselves can influence bacterial communities.

Study 2 reports that the internalization of dextran nanoparticles by probiotics can enhance the production of antimicrobial peptides *in vitro*. Furthermore, probiotics with enhanced antimicrobial activity can prevent pathogenic gut infections and change the composition of the gut microbiome *in vivo*. In conclusion, study 2 suggests the use of prebiotic nanoparticles with probiotics as an alternative to antibiotics.

Overall Conclusion

Due to the abuse of antibiotics, various problems have emerged including that bacteria have become resistant to antibiotics. Therefore, in the livestock and food industries, finding an alternative to antibiotics is needed. In this situation, probiotics are regarded as an alternative to AGPs in the livestock industry because they are regarded as safe. Moreover, probiotics can modulate the gut microbiota and circumstances to provide health benefits to the hosts. However, for probiotics to replace antibiotics, the inherent microbial activity of probiotics must be enhanced to maintain their ability in various hosts, which is challenging. Given that prebiotics enhance the growth and/or activity of probiotics, prebiotic nanoparticles were developed using the self-assembly method. Polysaccharides such as inulin and dextran were conjugated with hydrophobic residues including phthalic anhydride, acetic anhydride, and propionic anhydride. A probiotic was treated with the developed prebiotic nanoparticles. *Pediococcus acidilactici* (PA) was chosen as the probiotic bacteria, a probiotic which can produce an antimicrobial peptide called pediocin. Therefore, the purpose of this study was to reveal the effect of prebiotic nanoparticles on the probiotic activity of PA, unlike the effects of prebiotics or probiotics themselves.

In study 1, three types of prebiotic nanoparticles were developed using inulin and phthalic anhydride, acetic anhydride, and propionic anhydride. Phthalyl- (PINs), acethyl- (AINs) and propyl-inulin nanoparticles (PrINs) were synthesized. For the PINs, four type of nanoparticles were developed by varying the molar ratio

of phthalic anhydride. Based on the integration of the protons in the phthalic acid and the protons in inulin, the PINs were named as follows: PIN1 (content of phthalic acid: 9.9 mol.-%), PIN2 (15.2 mol.-%), PIN3 (20.4 mol.-%) and PIN4 (27.4 mol.-%). The morphologies and sizes were a smaller consequence of the higher content of phthalic acid groups. For the AINs and PrINs, the conjugated content of the hydrophobic groups was 78.8 mol.-% and 72.4 mol.-%. Initially, to check whether specific hydrophobic groups in the inulin nanoparticles could affect the potency of the antimicrobial activity of PA, various antimicrobial assays were used. The PINs, AINs, and PrINs increased the antimicrobial activity of PA. Especially, the antimicrobial activity was the highest when the PINs were used to treat PA compared to other groups. Moreover, the PINs were able to enhance the antimicrobial properties of PA against various pathogens including Gram-negative (*Salmonella* Gallinarum and ETEC K88) and Gram-positive (*Listeria monocytogens*) bacteria. By confocal microscopy and FACS analysis, it was determined that the PINs could be internalized into PA according to their sizes. The internalization of the PINs into PA was largely regulated by the glucose transporter in the probiotic while the fructose or galactose transporter was not involved. In addition, the internalization of the PINs was done in an energy-dependent manner. Interestingly, without a change in viable cell growth and lactic acid production, the PIN-treated probiotics enhanced the production of the antimicrobial peptide (pediocin) which is effective against both Gram-positive and Gram-negative pathogens. This could support that internalization of the PINs could enhance the antimicrobial activity of PA against pathogens. Particularly, the

antimicrobial activity of the PINs-internalized probiotics was about 9-fold higher than that of the untreated probiotics. Gene transcriptional analysis showed a rise in the pediocin activity in the PA internalized with the PINs accompanied with the enhanced expression of genes related to the stress response (*GroEL·ES*, *DnaK·J*, and *ClpB*) and to pediocin biosynthesis (*pedA* and *PedD*). Because the production of pediocin is known as a defense mechanism of PA, it seems that the higher expression of stress genes could reflect that the internalization of the PINs by PA causes mild stress activating the expression of pediocin.

In study 2, to demonstrate the previous results with a different type of prebiotic, nanoparticles were developed with dextran, denoted as phthalyl dextran nanoparticles (PDNs). Based on the integration of protons, the phthalyl content of the PDNs was 27.6 mol.-%; the size was 238.7 nm, and the zeta potential was -27.32 mV which was similar to the PINs. The PDNs had higher antimicrobial activities against both Gram-negative and Gram-positive pathogens as did the PINs compared with dextran or PA alone. Like the PINs, the PDNs were able to internalize into PA in various conditions. The PDNs were internalized in a time and temperature dependent manner like the dextran. Additionally, using three types of sugars (glucose, fructose and galactose), it was demonstrated that the PDNs internalization was largely controlled by the glucose transporter, while dextran diffused into the PA in all cases. Consequently, without any change in the growth and lactic acid production of PA, internalization of the PDNs enhanced the expression of the pediocin synthesizing genes by activating the PA defense system.

In addition, in study 2, the antimicrobial activity of PA internalized with the PDNs was proven in animal experiments. The PA internalized with the PDNs was able to suppress the infection of pathogenic bacteria in the gut. Physiological changes, such as body weight gain, feed intake, and colon length, showed that PA internalized with the PDNs could reduce the negative effects of pathogenic gut infection. Moreover, the composition of the gut microbiome was clustered by the supplementation and administration of the PDNs in the PA preventing the reduction in the diversity of the microflora. The enhanced antimicrobial activity of the PA internalized with the PDNs could also contribute to decreasing the number of pathogens (EHEC O157:H7 and *Proteobacteria*) and increasing the beneficial bacterial species related with prebiotics (*Bifidobacterium* spp., *S24-7*, and *Anaerostipes*) in mice. Additionally, the F/B ratio of the mice changed in the PA treated with the PDNs. The results from this study indicate that the higher pediocin production may suppress the pathogenic gut infection *in vivo*.

Overall, although the mechanism is not completely clear, it appears that the internalization of prebiotic nanoparticles (PINs and PDNs) by PA causes mild stress to activate the PA defense system, leading to an increased production of pediocin. The higher production of pediocin could have the effect of decreasing infection caused by pathogens in the animal gut. Moreover, the prebiotic nanoparticles (PDNs) could modulate the population of the gut microbiota. It can be inferred that prebiotic nanoparticles can act as prebiotics in the mouse digestive tract because prebiotic related bacteria were enhanced in the group that was treated

with PA with the internalized prebiotic nanoparticles. Based on the results from study 1 and 2, prebiotics in the form of nanoparticles can be an intracellular stimulator of probiotics, demonstrating a new avenue for the biological production of antimicrobial peptides and their potential use for alternatives to antibiotics in many gut diseases (Figure 43).

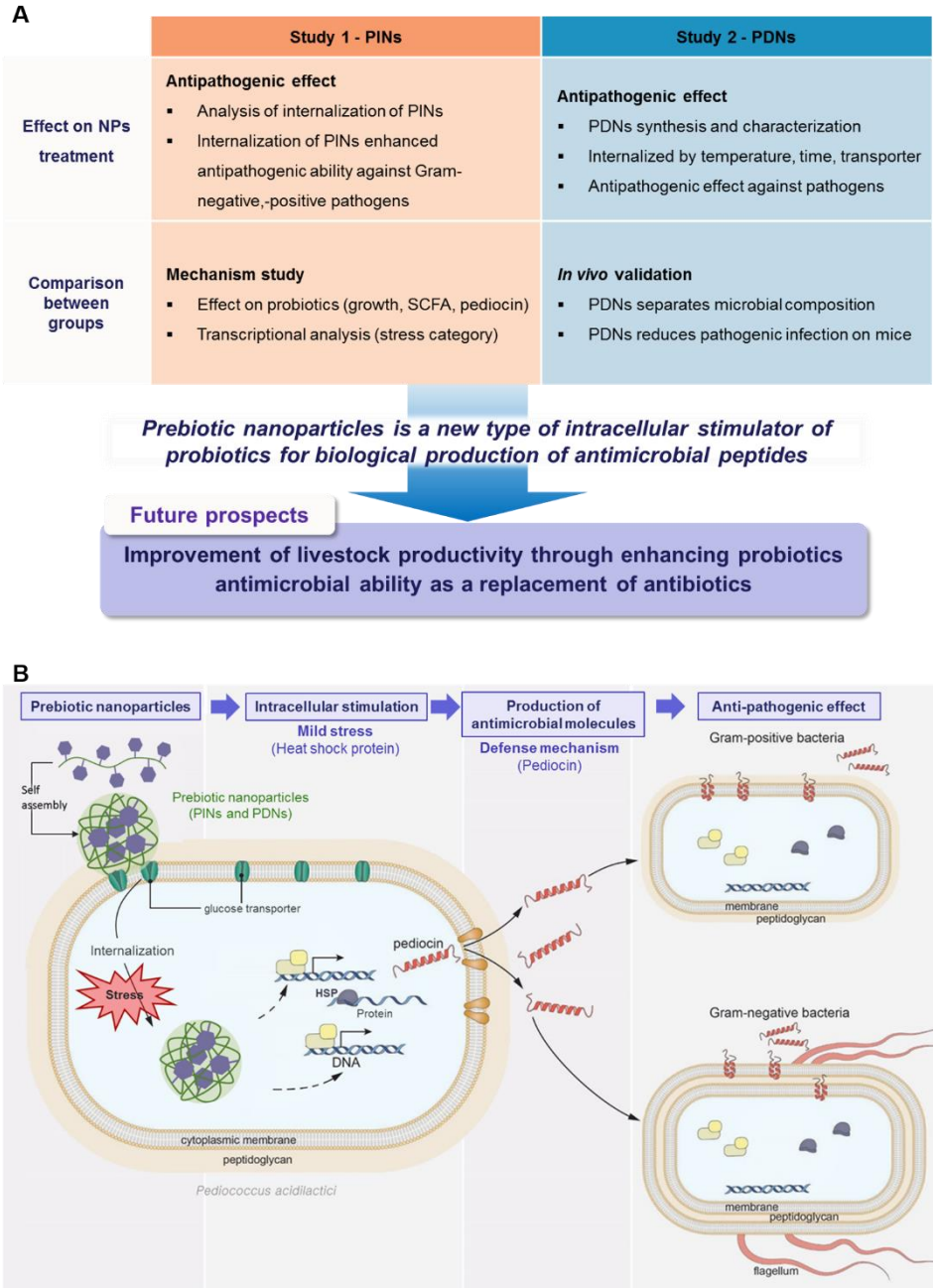


Figure 43. Overall conclusion of the study. (A) Summary of the study. (B) Schematic illustration of enhancing antimicrobial activity of PA by prebiotic nanoparticles.

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Summary in Korean

항생제는 의약, 식품 보존제, 축산 등 다양한 분야에서 이용이 되어져 왔다. 하지만, 항생제의 오남용으로 항생제 저항성 미생물의 수가 높은 수준까지 증가하였고 이로 인한 병원균 감염이 사회적으로 위협이 되고 있다. 이에, 축산분야에서는 성장촉진용 사료첨가용 항생제의 사용을 금지하였고 이를 대체 할 수 있는 항생제 대체제를 개발하고자 노력하고 있다. 항생제 대체제 중 하나로 생균제의 사용이 증가되고 있고 이에 관한 연구가 많이 이뤄지고 있다. 생균제는 살아있는 미생물로서 적정량을 급여하거나 섭취하였을 때 항균 능력과 장내미생물 균총을 변화시킴에 따라 건강증진 효과를 보인다고 알려져 있다. 다양한 생균제 중에서도 *Pediococcus acidilactici* (PA)는 다양한 환경에서 성장 할 수 있고, 다양한 형태의 항균 물질(유산, 박테리오신)을 분비함에 따라 여러 병원균(*Escherichia coli*, *Salmonella*, *Listeria*)에 대한 억제 효과를 지니고 있어서 식품이나 축산업계에서 많이 이용되고 있다. 하지만, 아직까지는 생균제 단독이 분비하는 항균물질의 양이 적어 항생제를 대체하기에는 어려움이 있다.

프리바이오틱스는 장내 미생물이나 생균제의 성장 및 기능을 증진시킬 수 있는 물질로서, 생균제와 함께 이용하여 생균제의 효능을 증진시키고자 많이 이용되고 있다. 더불어, 나노입자의 사용이 증진되고 있는데, 이는 나노입자가 다양한 물리적 생리적 장벽을 극복할 수 있는 특징을 지니고 있을 뿐 아니라, 몇몇 나노입자들의 경우 직접적으로 병원균에 효과가 있다는 것이 밝혀지고 있기 때문이다. 이에 본 연구에서는 프리바이오틱스를 프리바이오틱스

나노입자 형태로 바꾸어 사용함으로써, 생균제의 기능을 더욱 증진시켜 항생제 대체제로서의 기능을 높이고자 하였다. 두 가지 다당류인 이눌린과 덱스트란을 이용하여 두가지 종류의 프리바이오틱스 나노입자를 개발하였다. 프리바이오틱스 나노입자는 다당류인 이눌린과 덱스트란에 소수성 잔기들인 프탈기(phthalic anhydride), 아세틸기 (acetic anhydride), 프로필기 (propionic anhydride)를 결합하여 형성하였다. 이렇게 형성된 다양한 프리바이오틱스 나노입자들이 PA 에 미치는 영향을 *in vitro* 와 *in vivo* 에서 확인하였다.

Study 1 에서는 세가지 종류의 이눌린 나노입자를 합성하여 이들이 PA 에 미치는 영향을 분석하였다. 이눌린 나노입자는 프탈기– (PINs), 아세틸기– (AINs), 그리고 프로피올기– (PrINs)를 도입하여 형성하였다. 합성한 나노입자들을 PA 에 처리한 뒤, 항균능력의 변화를 측정하였을 때 나노입자를 처리한 모든 그룹에서 항균능력이 유의적으로 높아지는 것을 확인하였다. 특히, 세가지 나노입자 중에서 프탈기를 도입한 PINs 이 병원균에 대해 항균능력을 가장 높이는 것으로 확인되었다. 이에 PINs 이 PA 의 항균능력을 증진시키는 원인을 분석하고자 하였다. 먼저, 나노입자들은 세포 내로 도입(internalization)이 일어난다고 알려져 있기 때문에, PINs 이 PA 내부로 도입되는지 여부를 파악하였다. PINs 은 나노입자의 크기가 작을 수록, 온도에 따라 도입이 되는 것을 확인하였다. 더불어, 나노입자의 도입이 transporter 에 따라 달라지는지를 분석하기 위해 세가지 단당을 이용하여 분석을 진행하였을 때, 포도당 transporter 를 인지함으로서 도입이 된다는 것을 확인 할 수 있었다. 나노입자의

도입으로 PA 의 항균 물질 분비에는 어떠한 영향을 미쳤는지 보았을 때, PA 의 항균펩타이드인 pediocin 의 발현량이 유전적 그리고 단백질 수준에서 모두 증가하는 것을 증명할 수 있었다. 더불어, transcriptional 한 분석을 통해 나노입자의 도입이 pediocin 의 발현량을 증가시키는 메커니즘을 분석하고자 하였다. 그 결과, 나노입자가 도입된 그룹에서는 PA 단독에 비해 스트레스 관련 유전자들의 발현량이 유의적으로 높은 것을 확인할 수 있었고, 이는 나노입자의 도입이 PA 에 약한 스트트레스를 유발한다는 사실을 파악할 수 있었다. 즉, 나노입자의 도입이 PA 의 방어기작을 발동시킴으로써 pediocin 의 발현량이 증가하고, 그 결과 그람 음성균 (살모넬라, 대장균)과 그람 양성균 (리스테리아균) 모두에서 PA 단독 혹은 PA 에 이눌린을 처리한 그룹에 비해 항균능력이 유의적으로 높아진다는 알 수 있었다.

Study 2 에서는 프리바이오틱스의 종류가 변하여도 동일한 효과를 보일 수 있는지를 분석하기 위해, 덱스트란을 이용하여 나노입자를 형성하였다. 덱스트란 나노입자 (PDNs)를 PA 에 처리하였을 때 PA 의 항균능력이 PINs 와 마찬가지로 유의적으로 높아지는 것을 확인하였다. 앞서 study 1 에서 합성한 PINs 와 동일한 메커니즘을 통해 PDNs 의 항균능력이 증진되는지를 보기 위해 PDNs 를 이용하여 추후 실험을 진행하였다. PA 에 PDNs 가 시간, 온도, 그리고 포도당 transporter 를 인지하여 도입되는 것을 확인하였으며, 나노입자의 도입이 PA 의 항균펩타이드인 pediocin 의 발현량 증진에 영향을 미친다는 것을 알 수 있었다. 즉, PDNs 의 도입은 PINs 와 마찬가지로 PA 의 방어기작을 증진시킴으로써 pediocin 의 발현 수준을 유전적,

단백질 수준에서 높임으로써 그람 음성균과 그람 양성 병원균에 대해 PA 의 항균능력을 높일 수 있는 것을 확인 할 수 있었다. 더 나아가서, study 2 에서는 PDNs 가 *in vivo* 에서도 동일하게 항균 효과를 보이는지를 확인해보기 위해 동물 모델로 쥐를 활용하여 실험을 진행하였다. 다양한 병원균 중에서 EHEC O157:H7 을 모델 병원균으로 사용하여 동물실험을 진행하였을 때, PDNs 을 도입한 PA 가 병원균의 감염을 감소시킬 수 있다는 사실을 확인하였다. 병원균의 수는 줄이고, 프리바이오틱스와 관련된 유익균을 증진시킴으로써 병원균의 감염을 억제할 수 있음을 확인하였다. 더불어, PDNs 를 도입한 PA 를 처리한 그룹이 다른 그룹과는 다르게 장내미생물의 균총이 변화됨을 확인할 수 있었다.

Study 1 과 2 의 결과들을 통해 프리바이오틱스 나노입자의 도입이 생균제의 항균물질을 많이 분비할 수 있도록 하는 새로운 종류의 세포 자극 물질이며 이는 생균제의 대사를 변화시킴으로써 항생제 대체제로서 이용될 수 있을 뿐만 아니라 다양한 장내 질환을 치료하는 데에도 이용이 가능할 수 있다는 가능성을 제시하였다. 즉, 프리바이오틱스 나노입자를 이용하여 생균제의 항균능력을 증진시키는 것은 효과적인 가축 생균제의 개발 뿐 만 아니라 나아가서는 축산의 생산성 증진에도 기여할 것이다.

주요어: 프리바이오틱스, 생균제, 나노입자, 세포 내 도입, 항균펩타이드, 페디오신

학번: 2014-21915

Appendix. Development of oral delivery carrier of probiotics using pH-sensitive phthalyl inulin tablets

1. Introduction

Because of the prohibition of antibiotics, there has recently been a growing interest in the use of probiotics (Allen et al. 2013). This is because probiotics are generally considered as safe and confer health benefits to the host. Probiotics are used as an alternative for antibiotics and for treatment as anti-inflammatory drugs (Kechagia et al. 2013). An overdose of antibiotics and synthetic antibiotic drugs can cause side effects such as the production of antibiotic-resistant bacteria and antibiotic-associated diarrhea (Hansen et al. 2015). The probiotics produce antimicrobial molecules (e.g., lactic acid and bacteriocins) and enzymes, and this enables probiotics an alternative to antibiotics owing to producing antimicrobial effects toward pathogens, inhibiting pathogen colonization, modulating the immune system, and enhancing nutrient absorption (Kechagia et al. 2013). Among probiotics, *Lactobacillus* is the most common probiotic because *Lactobacillus* has shown an excellent antimicrobial activity against *Salmonella* spp. and *Escherichia coli*, which are major pathogens in livestock animals (Doyle and Erickson 2006; Forkus et al. 2017). The *Lactobacillus* reduces weight loss, improves feed intake and improves the growth performance of animals (Dowarah et al. 2017). In our previous study, we isolated *Lactobacillus reuteri* from porcine feces and selected

the highest antimicrobial effect on K88-positive *Escherichia coli* and *Salmonella enterica* subsp. (Lee et al. 2017).

To deliver probiotics orally, as one of the therapeutic drug or/and alternative of antibiotics, many strategies target the intestine as a major space for the delivery of probiotics to provide beneficial effect to the host (Akhgari 2015; Sinha and Kumria 2001). Since the intestine has a neutral pH, long transit time and reduced host enzymatic activity, an intestine-specific drug delivery system increases the bioavailability of probiotics (Ravi and Kumar 2008). However, the oral delivery of probiotics is extremely challenging because probiotics can get destroyed or/and cause cell death due to the acidic condition of the stomach (Papadimitriou et al. 2015). Therefore, delivering probiotics to the intestinal site safely by passing through the harsh gastric condition is an important aspect to have the probiotics a therapeutic effect to host. Recently, polymeric delivery systems have been attracted to deliver biological materials, proteins, genes, and chemotherapeutics because they can deliver the drugs to the target sites (Petros and DeSimone 2010). Among many strategies for orally delivering probiotics, the pH-sensitive polymers, such as hydroxypropyl methylcellulose phthalate (HPMCP) (Singh et al. 2015), hydroxypropylmethyl cellulose acetated succinate (Fukui et al. 2001), and cellulose acetate phthalate (CAP) (Lee et al. 2018) have been used to protect probiotics from harsh gastric condition since probiotics loaded into pH-sensitive polymers cannot be released at acidic pH condition due to deprotonated of carboxylic acids in the pH-sensitive polymers (Liu et al. 2017). However, these

pH-sensitive polymers only has the advantage on protecting the probiotics from harsh gastric conditions, therefore, we designed a new type of pH-sensitive polymers using inulin as prebiotics.

Inulin has been used as a prebiotic source in industrial applications because it can be found in many natural sources (e.g., chicory root, Jerusalem artichoke, leek, and onion) (Mensink et al. 2015). The inulin is consisted of fructose polymer linked by β (2→1) bonds containing glucosyl moiety at the chain terminal. Due to β (2→1) linkages in inulin, it is not digested by pancreatic enzymes in the upper GI tract (Mensink et al. 2015) although the gut microbiota can ferment inulin and produce short chain fatty acids (SCFAs) and can induce the growth of beneficial microorganisms, alter the composition of organisms in the gut microbiome and affect the host immune system (Seifert and Watzl 2007; Tremaroli and Backhed 2012). Also, there has been a growing interest in the use of inulin as an adjuvant or drug delivery system. Interestingly, the delta inulin as the microparticle form showed adjuvanting ability for enhancing immune activity in vaccines to the influenza, hepatitis B, etc (Skwarczynski 2017) while soluble inulin has less immunological activity (Petrovsky and Cooper 2015).

In industry, there are several methods on formulation of probiotics for administration of the feed such as powder, liquid, and spray forms although the most commonly used method is powder forms. However, there are not much reports on making a tablet form for oral delivery of probiotics although tableting probiotics with a pH-sensitive polymer successfully protected probiotics from

harsh stomach conditions and was easily able to formulate probiotics into tablet form by a previous study (Jiang et al. 2017).

In this study, we aimed to develop a new pH-sensitive tablet using phthalyl inulin (PI) to protect the probiotics from harsh gastric condition. As results, we obtained promising results for further *in vivo* application. To the best of our knowledge, this is the first report to exhibit the pH-sensitive property of PI to protect probiotics from harsh gastric conditions.

2. Materials and Methods

1) Materials

Lactobacillus reuteri LRT18 (LR, KCTC3594) used in this study was isolated from a previous study (Lee et al. 2017). All of 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 and MRS agar were purchased from BD Difco (Sparks, MD, USA) for the bacterial cultures.

2) Synthesis of phthalyl inulin (PI)

Phthalyl inulin (PI) was synthesized as described in a previous method (Kim et al. 2018). Briefly, 1 g of inulin (MW: 5000 g/mol) was dissolved in 5 mL of *N,N*-dimethylformamide and 2.0 g of phthalic anhydride were added in the above solution and 0.2 mL of 5% (w/v) sodium acetate was used as a catalyst. The reaction was conducted at 40 °C for 24 h under nitrogen gas. And then, the PI was dialyzed in cold water for 24 h. The PI was lyophilized and stored at -20 °C until use. The conjugation of phthalyl groups in PI was confirmed by 600 MHz 1H-NMR spectroscopy (AVANCE600, Bruker, Germany).

3) Tablet preparation

LR cultures were grown in MRS broth at 37 °C for 24 h and collected by centrifugation. Harvested cells were washed 3 times in phosphate buffer solution and suspended in 10% skim milk. The cells were then frozen at −20 °C for 12 h and lyophilized. The lyophilized probiotics were ground into a fine powder and stored at 4 °C until use. The tablets were prepared at room temperature (RT) by direct compression using a single press. For the tablets, a mixture of LR and PI (weight ratio of LR to PI= 1:1) was filled into a 4 mm diameter die. The tablets were formed under different pressures ranging from 3 to 10 kilopascal (KP) with a plane surface according to Tao *et al.* (Jiang et al. 2017).

4) Measurement of the probiotic (LR) viability and disintegration time of tablets

The viability of LR in the tablet was expressed as colony forming units (CFU). Briefly, the tablets were broken and dispersed in 1 mL of phosphate buffer solution (PBS, pH 6.8). And then, the serial-diluted suspension was dropped into the MRS agar plate and incubated at 37 °C to count the LR colonies according to Tao *et al.* method [22]. The tablets were transferred into 5 mL PBS (pH 6.8) and the complete disintegration time was measured.

5) Measurement of the swelling ratio of tablets

The tablets were transferred into 5 mL simulated gastric fluid (SGF) adjusted to pH 2 with pepsin (1000 unit/mL). The swelling ratio was calculated by the following equation (Chavda and Patel 2011).

$$Q = (Ms-Md)/Md$$

The swelling ratio is Q, the Md is the tablet mass in the dried state and the Ms is the mass of the tablet in the swollen state. At the beginning of the experiment, the excessive water outside the tablet was removed.

6) Stability of the tablets in the SGF with or without pepsin

The stability studies were performed as described in a previous method (Jiang et al. 2017). The SGF was prepared by PBS adjusted to pH 2.0 with or without pepsin (1000 unit/mL) by 1 M HCl. The tablets and LR powder were transferred into 5 mL of SGF with or without pepsin. The survivability of LR was observed as the CFU at the end of the incubation period (0, 30, 60, 90, 120 min) when incubated at 37 °C with 100 rpm.

7) Viability of the tablets in SGF and simulated intestinal fluid (SIF) medium in sequential exposure

The cell viability of LR in the tablets sequentially exposed to SGF and SIF was performed by the following method (Jiang et al. 2017) with some modifications. Tablets were incubated in 5 mL SGF (pH 2, 1000unit/mL pepsin) at 37 ºC with 100 rpm for 2 h. Then, the tablets were quickly transferred to 5 mL SIF and incubated at 37 ºC with 100 rpm for 4 h. SIF was prepared by PBS adjusted to pH 6.8 with 1.2% (w/v) bile salt. The viable cells were counted in the supernatant medium as well as non-disintegrated tablets at each incubation time.

8) Tablet stability

The stability of the tablets was tested when stored at 4 ºC for up to 6 months. Every month the cell viability was counted as described above.

9) Statistical analysis

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

3. Results

1) Synthesis and characterization of PI

To develop a pH sensitive polymer, the phthalic group was introduced to inulin by an ester bond between hydroxyl groups in inulin and carboxylic acids in phthalic acid. The reaction scheme of the synthesis of PI is shown in Figure 1A. After synthesizing the PI, the degree of the phthalic groups in the PI was estimated by measurement of 1H-NMR. The fifth protons of inulin appeared at 3.8 ppm and the protons of the phthalic groups in the PI appeared at 7.4-7.7 ppm as shown in Figure 1B. Based on the integration of protons on both the inulin and phthalic groups, the degree of the phthalic groups in the PI was 36.4 mol.-%.

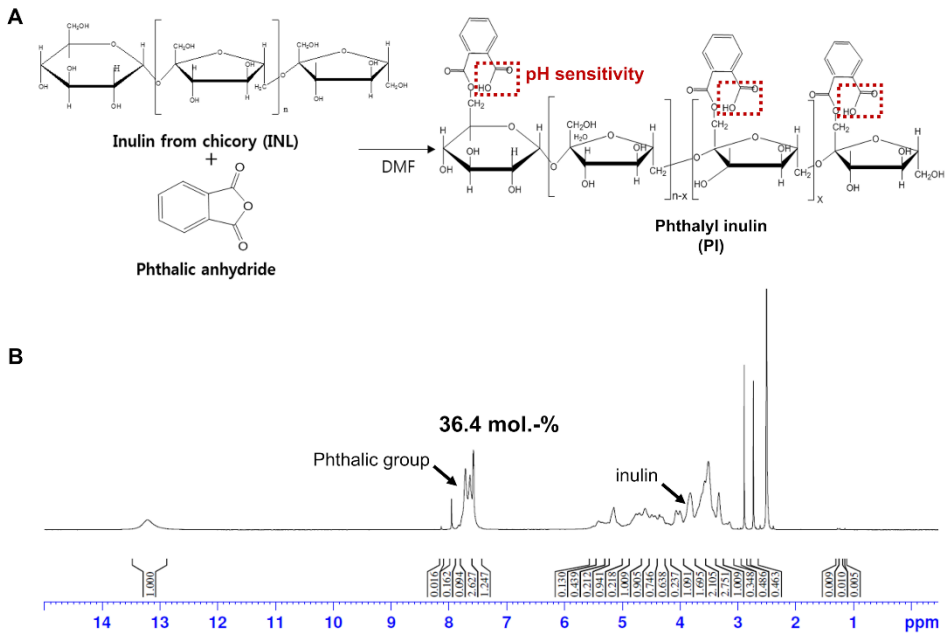

Figure 1. Scheme and characterization of phthalyl inulin (PI). (A) Chemical reaction scheme of PI and (B) NMR spectrum of PI

2) Effects of compression forces on the viability of LR and tablet properties

To evaluate whether a different compression force can affect the viability of LR, the viability of the LR was measured after tableting. There were no significant differences on the viability of LR in the tablets among used compression forces (Figure 2). To determine the protection effect of the LR in the gastric conditions, the swelling ratio of the tablets and viability of the LR in SGF were measured. The swelling ratio of the tablets prepared according to different compression forces was very low in SGF condition (Figure 3). It was observed that the tablets were not completely disintegrated within 2 h in the gastric condition. Specially, among the groups, the highest compression force (15 KP) showed the least swelling ratio. The viability of LR in the gastric condition was then measured using the SGF condition with or without pepsin (Figure 4). The 5, 10, and 15 KP tablets and free probiotics (powder) were loaded in SGF for 2 h. The results showed that the viability of the free probiotics dramatically decreased in both the SGF condition and especially in the presence of pepsin. However, LR-loaded PI tablets were able to protect probiotic death in the SGF regardless of the presence of pepsin. The viability of LR between tablets and free LR showed significant differences in SGF in the presence of pepsin after 2 h (Figure 4B), suggesting that PI tablets was able to protect LR from the harsh stomach condition. Furthermore, the higher compression force increased the viability of the LR in the gastric condition. The LR viability in the 15 KP tablets was significantly higher than free

LR in SGF without a pepsin at 30 min (Figure 4A). In SGF with a pepsin, the difference in viability of LR between free LR and the loaded LR in the 15 KP tablet was significantly higher at 60 min (Figure 4B). Moreover, the viability of LR loaded in the 15 KP tablet was significantly higher than LR loaded in the 5 KP and 10 KP tablets after 2 h especially in the presence of pepsin, indicating that higher compression force was able to protect probiotics better in the gastric condition.

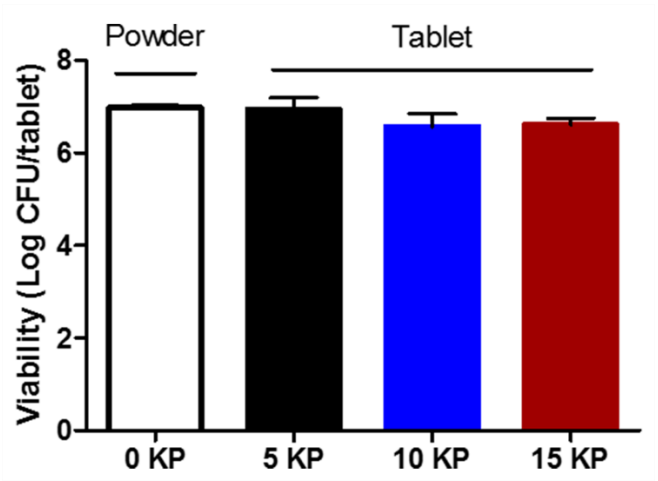

Figure 2. Viability of LR after tableting. Viability of *Lactobacillus reuteri* (LR) after tableting under different compression forces (5, 10, and 15 KP). (means ± standard deviation, SD; n=3).

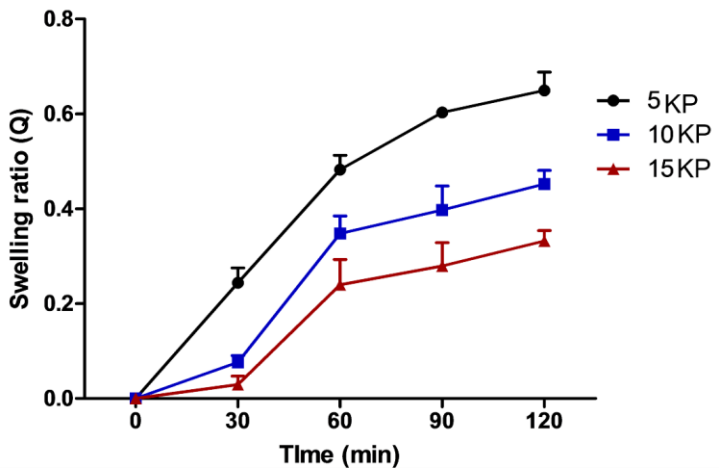

Figure 3. Swelling ratio of LR-loaded PI tablet in SGF. Swelling ratio of LR-loaded PI tablets with different compression conditions (5, 10, and 15 KP) until 2 h incubation in SGF (means ± standard deviation, SD; n=3). (PI: phthalyl inulin, LR: *Lactobacillus reuteri*, and SGF: simulated gastric fluid)

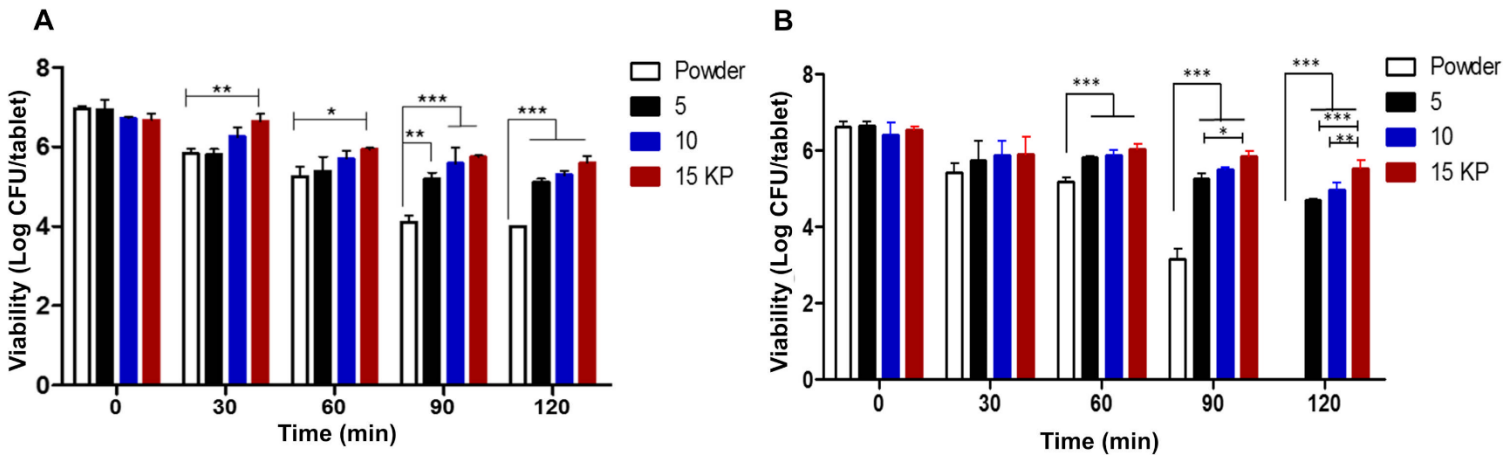

Figure 4. Survivability of LR in LR-loaded PI tablets in SGF. Survivability of LR in LR-loaded PI tablets in SGF (pH 2.0) without pepsin (A) and with pepsin (B) until 2 h at 37 °C (means ± standard deviation, SD; n=3). (PI: phthalyl inulin, LR: *Lactobacillus reuteri*, and SGF: simulated gastric fluid)

To investigate the swelling effect in the intestinal fluid, the time for complete disintegration in SIF was measured among the different compression forces. In Figure 5, the disintegration time increased with an increase of the compression force. The disintegration time for 15 KP was 160 min; however, for 10 KP it was nearly 110 min and for 5 KP it was 50 min or less. This points out that tablets fully disintegrates in intestinal condition due to the pH sensitivity of the PI and the compression force also affected the disintegration ability of the pH-sensitive tablets.

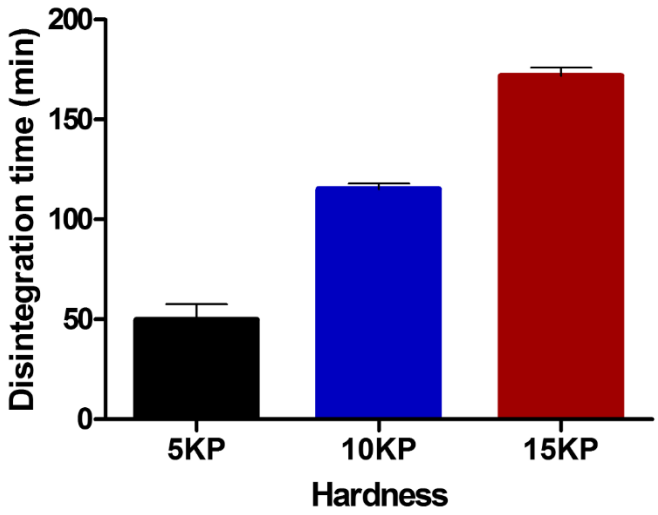

Figure 5. Disintegration time of LR-loaded PI tablets in PBS. Disintegration time of LR-loaded PI tablets with different compression forces in PBS (pH 6.8) (means ± standard deviation, SD; n=3). (PI: phthalyl inulin, LR: Lactobacillus reuteri, and PBS: phosphate buffer solution)

3) Release and viability of LR from LR-loaded PI tablets in SGF and SIF

The release and cell viability of LR from LR-loaded PI tablets in SGF and SIF were tested by sequentially immersing the tablets into SGF and SIF. As shown in Figure 6A, the LR released from the tablets in SGF and SIF was calculated. In SGF, no viable released cells were found from the tablets. In SIF, the 5 KP and 10 KP tablets released viable cells faster than the 15 KP from the tablets. The higher compression force tablet delayed the release of LR from the tablets compared to the other two tablets. However, nearly all of the probiotics were released from the tablets of all groups after 5h. The viability of LR was measured by sequentially exposing the tablets to SGF and SIF (Figure 6B). When the tablets were exposed to SGF, the LR viability became decreased with time. Although the LR viability slightly changed in SIF after 5h, more viable cells remained inside the tablets prepared with the higher compression force than the lower compression force. The viability of LR inside 15 KP tablet group was significantly higher than the other two groups after 7 h in SGF and SIF conditions. Altogether, owing to the pH-sensitive property of the used PI, PI tablets were able to protect LR from harsh gastric condition and release LR in intestinal condition, while PI itself does not affect the LR viability when the PI tablets are disintegrated.

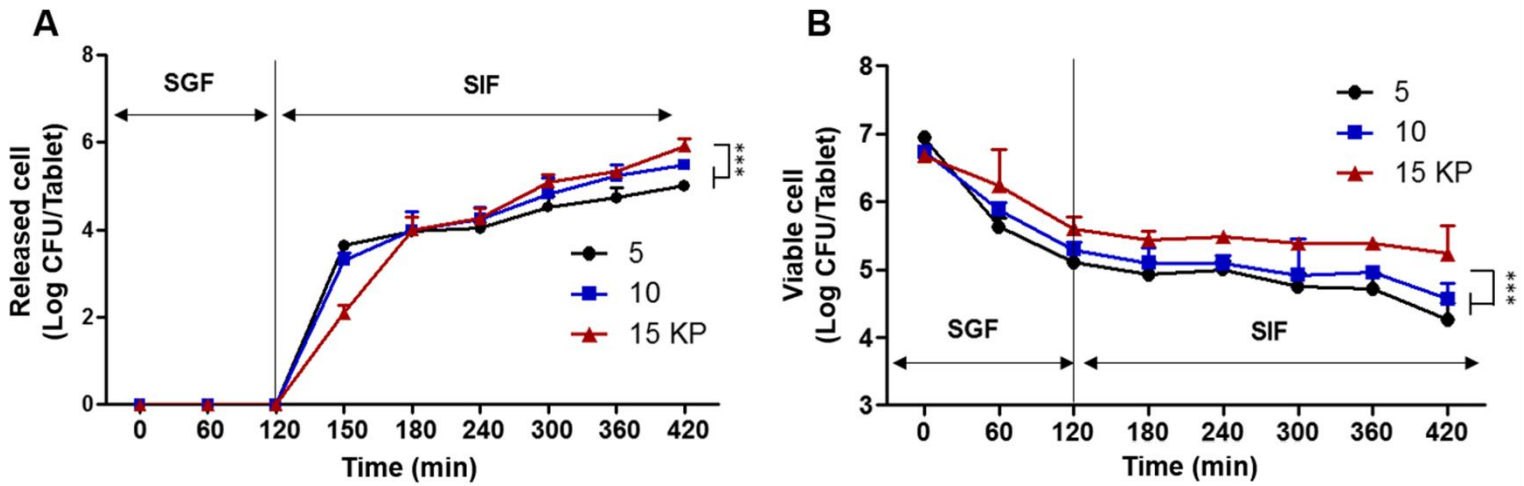

Figure 6. Release and viable cells in SGF and SIF. Released (A) and viable cell numbers (B) of LR from LR-loaded PI tablets sequentially exposed to SGF and SIF until 7 h at 37 °C (means ± standard deviation, SD; n=3). (PI: phthalyl inulin, LR: Lactobacillus reuteri, SGF: simulated gastric fluid, and SIF: simulated intestinal fluid)

4) LR viability in LR-loaded PI tablets for long term storage

The stable viability of probiotics is a major index for industrialization of probiotics. The stability of LR inside the tablets for storage were checked during 6 months at 4 °C. The temperature was selected because most probiotic products recommend storage in a refrigerator. In Figure 7, the stability of LR for 6 months was calculated by the viable CFU per one tablet. Free probiotics as a powder form dramatically decreased after 3 months. However, the viability of the LR in tablet groups were more significantly stable after 6 months. In particular, higher compression force tablets (10 and 15 KP) showed significantly higher viable cells at the end of 6 months than the 5 KP tablet. Overall, the PI-based tablets were able to stabilize the viability of LR by tableting and higher compression force also showed the higher cell stability.

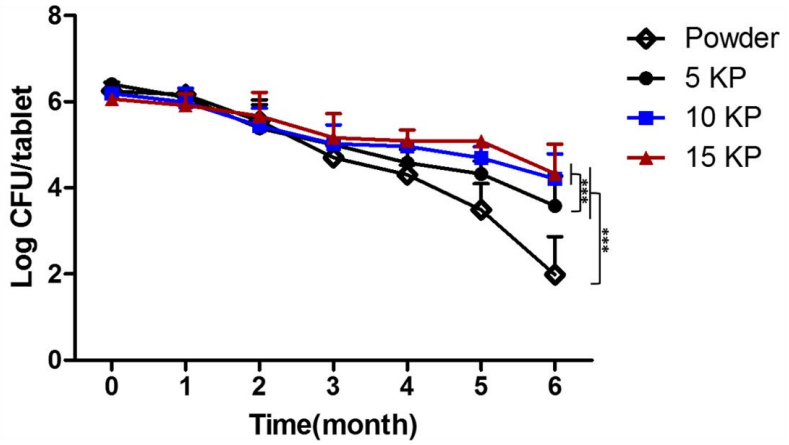

Figure 7. Stability of tablets. Storage stability of LR in LR-loaded PI tablets during 6 months at 4 °C (means ± standard deviation, SD; n=3). (PI: phthalyl inulin and LR: *Lactobacillus reuteri*)

4. Discussion

In this study, we developed a new pH-sensitive tablet using the PI and examined the effect of pH-sensitivity by PI on probiotic survivability and stability for oral delivery of probiotics using pH-sensitive PI tablets. In delivering probiotics to the host gut, one of the most important aspects is to permit the probiotics to survive after passing through harsh gastric conditions (Solanki et al. 2013). Generally, most probiotic products in the market for human use are now selling as liquid or semi-liquid form containing probiotics and prebiotics together (Collins JW 2009). Also, the products are just a mixture of probiotics and prebiotics, which shows low cell viability after oral ingestion because prebiotics cannot protect probiotics from harsh gastric conditions in the form of the mixture (Collins JW 2009). Furthermore, the probiotics used for livestock animals are administered orally through feed although most probiotics have been just added in feed without any protection and the amount of the probiotics used is very inconsistent from animal to animal (Cheng et al. 2014). Therefore, developing a dry form of probiotics that can protect probiotics from harsh stomach conditions and homogenous administration of the probiotics through the oral route are needed. For these reasons, we designed a new pH-sensitive PI to protect probiotics in low pH conditions and to form homogenous tablets as the dry form for oral administration. The PI was prepared by conjugation with phthalic anhydride with inulin through ester bond linkage because the remained carboxylic acid groups in phthalic acids after conjugation reaction have pH-sensitive property due to the deprotonation at

pH 7 and protonation in low pH such as pH 2 (Yang et al. 2011), which is similar with CAP or HPMCP used for the popular oral delivery system (Dai et al. 2004; Singh et al. 2015; Wang and Zhang 2012). Also, the inulin has been widely used as a prebiotic for many decades and also used in drug delivery systems for intranasal, parenteral, intravenous, and subcutaneous routes of administration (Imran et al. 2012) although the inulin itself is difficult to use as a carrier for the oral colonic drug delivery system because inulin is highly soluble in water. Therefore, many strategies have been tried to reduce the solubility of inulin in water by mixing hydrophobic coating materials such as Eudragit (Akhgari et al. 2006; Van den Mooter et al. 2003) or by conjugating hydrophobic residues (Castelli et al. 2008). By conjugation of the phthalyl groups to inulin, we were able to reduce the water solubility of inulin and protect probiotics from the low pH condition after making tablets.

First, we investigated the LR survival during tableting under different compression forces. There was no significant differences in probiotic viability among the different compression forces, which is similar to previous results (Calinescu et al. 2005; Jiang et al. 2017). Although the LR viability for the 15 KP tablet was most protection effect of LR through 2 h of incubation in the SGF condition. For the incubation in SGF with or without pepsin, the survivability of LR in the 15 KP tablet was both approximately 7 Log CFU. Especially, the LR viability loaded in 15 KP tablet was significantly higher than other tablet groups in SGF with pepsin. The results suggest that when tableting probiotics, the

compression force is one of the important parts to protect probiotics because a high compression force prohibits the fluid from physically coming inside the tablets. Moreover, the results were also consistent with the different disintegration time and swelling ratio between the groups. For the disintegration of the tablet in pH 2, figure 3 showed the swelling ratio of the tablets in pH 2. It was found that the swelling ratio of tablets were different according to the compression forces although the swelling ratio in all three groups was very low in SGF condition for 2 h incubation, suggesting that they showed very low swelling ratio due to the pH sensitivity of the tablets. Furthermore, in Figure 5, the disintegration time of tablets in SIF condition differed from their compression forces. Higher compression forces took more time for the tablets to disintegrate. The results were consistent with the release behavior in SIF condition in Figure 6, which 15 KP tablet released LR more slowly than 5 or 10 KP tablets for more than 60 min after changing to SIF condition. However, total viable cells released from the tablets were significantly higher in 15 KP than 5 or 10 KP because the survival rate of LR in 15KP tablets were higher after incubation in SGF condition.

To check pH-sensitivity of the PI, we incubated the PI tablets in the SGF media for 2 h with or without pepsin and compared with free LR (powder). Also, we exposed the PI tablets sequentially to SGF and SIF media. For identifying the survivability of probiotics in the gastrointestinal tract, most researchers choose pH 1.5-2 for gastric condition and pH 6.8-7.2 for the intestinal condition because the presence of pepsin and low pH are required for the stomach condition, when

testing the survivability of probiotics (Lian et al. 2003; Sahu et al. 2008). Although the pH of the stomach slowly declines from the neutral pH to pH 2 when food is ingested (Debas 1977; Holzapfel et al. 1998), pH of the stomach in fasting condition can be declined to pH 1.5, which suggests that acidic condition is one of the most challenging effects for the probiotics survival when administered by orally (Masco et al. 2007). Also, it has been generally reported that the ingested food remains in the stomach for 2-3 h and transits to the intestinal tract and remains for 5-12 h (Sorensen et al. 2001) although bile salt may have an antimicrobial effect toward bacteria, the intestinal pH is known to be pH 6.8-7.2, which is more suitable for bacteria to survive (Masco et al. 2007). Therefore, tablets should protect probiotics in gastric condition throughout this time and releases probiotics in the intestinal condition. The survivability of LR in the PI tablets was significantly higher than free LR from 30 min incubation in SGF media with or without pepsin, indicating PI tablets were able to protect LR from acidic condition. The swelling degree of PI tablet in SIF was higher than in SGF due to the pH sensitivity of the PI. Moreover, the viability and release behavior of LR in SGF and SIF was shown to be similar to other types of pH-sensitive tablets (Anal and Singh 2007), such as in SGF the viable release cells were not shown and the fast release of probiotics was shown in SIF as the swelling degree of PI increases at pH 6.8. However, our study may have limitations in *in vitro* condition because it is very difficult to mimic the *in vivo* condition by adjusting the pH with enzymes and the pH in the gastric compartment actually follows a slow decline from neutral to pH 2 over the course of 3 hours when ingested with a meal.

Therefore, a more study on the protection and release of LR from the new type of pH-sensitive tablets should be conducted *in vivo* in a near future.

LR-loaded PI tablets also increased the LR stability for long-term storage compared to free LR. The results suggest that PI tablets can be used in industry since many probiotics are stored at 4 °C for more than three months. In conclusion, the PI is a suitable material for making probiotic tablets that can preserve cells in harsh gastric conditions, release easily in the intestinal condition and show long-term stable storage. To the best of our knowledge, this is the first report to suggest the possibility of PI as a tableting material and to be used as an alternative to antibiotics in industry.

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