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Optimization of production process for industrialization of probiotics feed additives as antibiotic alternatives

사료첨가용 항생제 대체 생균제의 산업화를 위한 생산공정의 최적화

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Summary

As a kind of growth promoter, antibiotics have been used to improve the feed efficiency and production performance of livestock since the 1940s. However, the abuse of antibiotics also has caused a variety of side effects, such as the emergence of antibiotic resistant 'super bacteria', damage to beneficial intestinal bacteria, disorders and so on. After recognizing the gravity of these situations, many countries have promulgated laws prohibiting the use of antibiotics in the livestock breeding industry. Therefore, banning the addition of antibiotics to animal feed and developing antibiotic substitutes have become one of the most urgent problems to be solved in the animal industry currently.

Probiotics are considered to be an effective substitute for antibiotics and one of the options for improving livestock production. It can have a beneficial impact on the health of the host when ingested in an appropriate amount. Acute diarrhea is a major problem after weanling in young pigs, which is mainly caused by enterotoxigenic *Escherichia coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC). *Lactobacillus plantarum* is a probiotic bacteria which is widely used in food and medicine. It has a certain inhibitory effect on a variety of

gram-negative pathogens represented by *Escherichia coli*. A mutant of *Lactobacillus plantarum* used in this study was developed by genome shuffling technology and was named LP-GS1. Compared with the wild type, it has a stronger antibacterial ability against *Escherichia coli* K99, which is one of the pathogenic bacteria that cause pig diarrhea. Therefore, if it is possible to produce this kind of probiotic in large quantities at a relatively low price, it will be very meaningful for the development of the pig industry. However, lactic acid bacteria have relatively harsh requirements for growth conditions and nutritional elements, and this requirement is species-specific. Therefore, to achieve its industrialization, these problems must be solved first.

Study 1 is divided into two parts. In study 1-1, to mass produce a large number of LP-GS1 cells, the culture process was optimized. First, the one factor at a time (OFAT) method was used to select the factors that had the most significant effect on the growth of bacteria in broth which was determined to be the carbon and nitrogen source. Then, the efficiency of the mass production process and the concentration of different carbon and nitrogen sources were evaluated. Finally, in order to consider the interaction between the nutrient elements at the same time and to ensure the highest number of bacteria and the

lowest production cost, the RSM analysis method was used to model and predict the optimal concentration ratio of the nutrient elements. After optimizing the composition of the culture medium, to further improve the yield, the culture conditions, such as the temperature, inoculum size, culture time and initial pH, were optimized by the same analytical method. Finally, the growth medium for LP-GS1 (6.72% molasses and 1.21% yeast extract) and the growth conditions (temperature, 34.85 °C; inoculation volume, 1.29%; incubation time, 20.89 h and initial pH, 6.73) were obtained, and the total viable cell number obtained was around 4.75×10^9 cfu/ml. In study 1-2, to improve the survival rate of the LP-GS1 in the freeze-drying process, the use of a cryoprotectant was also optimized. First, the OFAT and PBD methods were used to evaluate the protective efficiency of various cryoprotectant candidates, and then, the CCD method in RSM was used to predict the optimal proportion for the selected protective agents. At this point, study 1 completed optimizing the conditions for the mass production of LP-GS1.

In study 2, the possibility of LP-GS1 as a feed additive, which could replace antibiotics, was verified by an *in vivo* piglets feeding experiment. During the five-week trial, the piglets supplemented with LP-GS1 showed similar growth

performance-related parameters to the positive control group. From the 2nd week, the body weight (BW) and the average daily gain (ADG) in the LP-GS1 supplementation group were higher than those in the other groups, but there was no significant difference. In addition, the incidences of diarrhea and death in piglets were observed throughout the experiment. It was found that the incidence of diarrhea was significantly lower than that of the control group after the addition of probiotics. The bacterial content in the feces of the weaning piglets was also evaluated by CFUs and DNA levels. The results show that compared with the negative control group, the beneficial bacteria (*Lactobacillus spp.*, *L.casei, L.acidophilus*, and *Bifidobacterium spp.*) were increased in the feces of the piglets, and the pathogenic bacteria (*Escherichia coli*) were decreased.

The results of study 1 and 2 show that LP-GS1, as a probiotic, could quite possibly achieve industrial mass production at a relatively low price. When it is supplemented in livestock as a feed additive, it can help to adjust the balance of the intestinal flora and improve the production efficiency of livestock. Therefore, as a substitute for antibiotics, LP-GS1 has great development value and a broad utilization space.

Key words: *Lactobacillus plantarum* GS1, mass production, optimization,

response surface methodology, bacterial diarrhea, weaning piglet

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List of Abbreviations

BW: Body weight

BUN: Blood urea nitrogen

CCD: Central composite design

CFU: Colony forming units

CPA: Cryoprotectant

DNA: Deoxyribonucleic acid

E. coli: Escherichia coli

ETEC: Enterotoxigenic *Escherichia coli*

EHEC: Enterohemorrhagic *Escherichia coli*

EIF: Entracellular ice formation

FCR: Feed conversion ratio

GC: Guanine-cytosine

gDNA: Genomic DNA

IIF: Intracellular ice formation

LAB: Lactic acid bacteria

L. casei: Lactobacillus casei

L.acidiphilus: Lactobacillus acidophilus

LDL: Low-density lipoprotein

LP 177: *Lactobacillus plantarum* 177

LP-GS1: *Lactobacillus plantarum* genome shuffling 1

MRS: De Man, Rogosa and Sharpe

NCBI: National Center for Biotechnology Information

NEFA: Non-esterified fatty acids

OFAT: One factor at a time

PBD: Placket-Burman design

qPCR: Quantitative real-time PCR

RSM: Response surface methodology

SCFA: Short chain fatty acid

Subsp.: Subspecies

spp.: *Species pluralis*

WHO: World Health Organization

Introduction

Since the discovery of the promoting effect of antibiotics on animal growth in the middle of the 20th century, antibiotics have been widely used as feed additives in aquaculture and production. Low doses of antibiotics make farm animals more feed efficient, presumably by reducing mild infections and thereby reducing disease (Ferber 2003). However, the biggest problem with antibiotics is that it promotes resistance to drugs in some bacteria, or their resistance genes enter the human intestines in various ways and propagate them to the gut microflora in a variety of ways (Ferber 2003). Since 2006, the European Union (EU) banned the use of antibiotics as growth promoters or food additives (Alagawany et al. 2018). Since 2011, the Republic of Korea also banned the use of antibiotics in the animal industry. Therefore, probiotics have an important role as the best substitute for antibiotics because they not only inhibit the growth of pathogenic bacteria but also work as growth enhancers in humans and animals including poultry and fish.

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Radha Krishna Rao 2013).

Lactobacillus plantarum is an important species in the fermentation of various plant products (Ashenafi and Busse 1991), is known to produce antimicrobial substances, like plantaricin, that are active against certain pathogens, and is used as a probiotic (Ashenafi and Busse 1991). Recent studies have also shown that the modulation of the gut microbiota is a new beneficial target related to host health by affecting its immune system. With the increasing popularity of probiotic products among consumers, large-scale fermentation of lactic acid bacteria such as *Lactobacillus* is gaining importance (Liew et al. 2005).

Because lactic acid bacteria have very demanding growth conditions, their growth is affected by pH, temperature, medium formulation and other fermentation conditions. Moreover, the nutritional requirements differ depending on the *Lactobacillus* species or strain. Thus, even though the MRS (deMan, Rogosa, and Sharp) medium introduced by de Man et al. has been widely used as a commercial medium for *Lactobacillus* species, it is not sufficient to maximize the growth of a specific strain type. In addition, freeze-drying is commonly used to stabilize lactic acid bacteria. Many factors have been reported to influence freeze-drying survival, including bacterial species, cell density, lyoprotectant, freezing rate, and other process parameters.

Therefore, to realize the mass production of specific lactic acid bacteria, it is necessary to optimize the above conditions.

Response surface method refers to a series of deterministic experiments using polynomial functions to approximate implicit limit state functions. By reasonably selecting test points and iterative strategies, the polynomial function can converge to the failure probability of the real implicit limit state function. It consists of a group of mathematical and statistical techniques used in the development of an adequate functional relationship between a response of interest, y, and a number of associated control (or input) variables denoted by x_1, x_2…. x_k. It is a powerful test technique for multilevel factors because it requires fewer practical laboratory tasks compared to studying one factor at a time. Response surface methodology (RSM) has been widely used in many fields, including biotechnology, and is suitable for studying the effects of different factors on the mass production of lactic acid bacteria (Malheiros et al. 2015).

The aim of this study was to optimize the mass production process of *Lactobacillus plantarum* GS1 by response surface methodology to improve its industrial value. To reduce the production cost while still ensuring a high

density culture, the composition of the culture medium and culture conditions were optimized first. After that, to improve the survival rate and storage stability of the freeze-drying process, the use of cryoprotectants were also optimized. In addition, the role of probiotics was verified by *in vivo* experiments, and the effect of the probiotics as an antibiotic substitute on animal intestinal microflora was also analyzed.
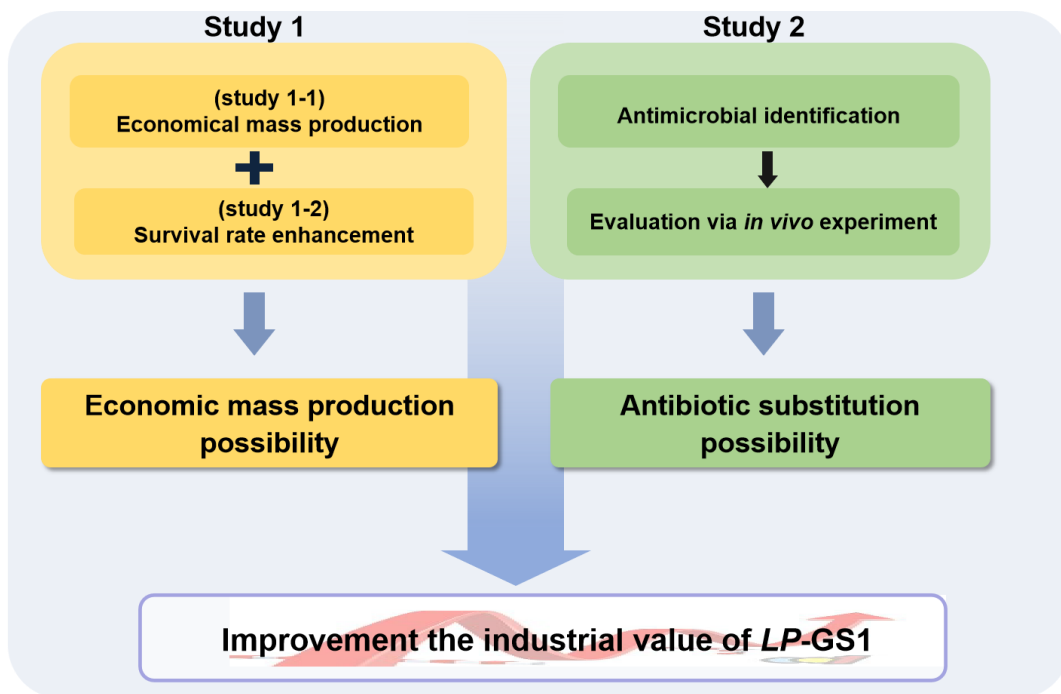
Figure 1. Aim of the study and research organization.

Review of Literature

1. Antibiotics

1) Development and problems of antibiotics

Antibiotic is a class of secondary metabolites produced by microorganisms (including bacteria, fungi, actinomycetes) or higher animals and plants in the process of life, which can interfere with the development function of other living cells. At present, the commonly used antibiotics are the extract from the culture liquid of genetically modified engineering bacteria and the compounds synthesized or semi-synthesized by chemical methods. The modern era of antimicrobial therapy started with the clinical use of sulfonamide in 1936 (Soares et al. 2012). Since then, antibiotics have been widely used all over the world. The "golden age" of antibiotics began with the production of penicillin in 1941, when this compound, discovered by Alexander Fleming in 1928, was finally mass-produced and first made available for limited clinical trials. Moreover, the invention of antibiotics made revolutionary changes in medicine in the 20th century. Because they are easy to obtain and can effectively inhibit the reproduction of pathogenic

bacteria, the situation of overuse is very serious, especially in livestock

industry (Kohanski, Dwyer and Collins 2010).

However, the abuse of antibiotics has caused very serious problems. The

most serious problem is the emergence of resistance bacteria and its spread

worldwide which may have a great impact on economic and social development

(Laxminarayan et al. 2013). The decreasing effectiveness of antibiotics in

treatingcommon infections has quickened in recent years, and we are at the

dawn of a postantibiotic era (Jesse T. Jacob 2013). In health care environment,

drug-resistant bacteria spread rapidly and have serious consequences for

vulnerable hosts (Laxminarayan et al. 2013). Hospital data from developing

countries show that 71% of *Klebsiella spp.* and 50% of *E.coli* which are

pathogens that cause neonatal infections, are resistant to gentamicin. In Pakistan,

the emergence of pan-resistant bacteria such as *Acinetobacter spp.* and

carbapenem-resistant enterobacteriaceae has made these infections the cause of

sepsis and untreatable (Perry et al. 2011).

2) **Antibiotic resistance of bacteria**

The so-called drug resistance of bacteria refers to the reduction or even

disappearance of the sensitivity of bacteria to drugs after repeated contact with

drugs, resulting in the reduction or even ineffective of the curative effect of drugs on drug-resistant bacteria. Some bacteria are inherently resistant to some antibiotics, but they can also be resistant to antibiotics through chromosome gene mutations and horizontal gene transfer. (Blair et al. 2015). The inherent resistance of bacteria to antibiotics is caused by their inherent structural or functional characteristics. Recent studies have found that many intrinsic resistance genes are resistant to different types of antibiotics, including β-lactam, fluoroquinolones and aminoglycoside (Blair et al. 2015).

There are great differences in the composition of cell membrane between Gram-negative bacteria and Gram-positive bacteria. The inherent resistance of some Gram-negative bacteria to many compounds is due to the inability of these drugs to pass through their outer membranes (Tsuchido and Takano 1988). Recently, the mechanism of resistance of bacterial biofilms to antibiotics has begun to be clarified: the first assumption is that some antibiotics have the possibility of slow or incomplete entry into the biofilm. For example, ampicillin can penetrate through a biofilm formed by a β-lactamase-negative strain of κ-pneumoniae but not a biofilm formed by the β-lactamase-positive wild type strain of the same micro-organism (Anderl, Franklin and Stewart 2000). The

second hypothesis is the change of chemical microenvironment in biofilm. When the difference of pH inside and outside the biofilm is greater than 1, it may hinder the entry of antibiotics. The third conjecture mechanism is that the subpopulations of micro-organisms in the biofilm can form a unique, highly protected cell differentiation similar to spore formation to protect themselves (Stewart and William Costerton 2001).

In addition to intrinsic resistance, bacteria can also acquire or develop resistance to antibiotics. This can be mediated by three main mechanisms: first, those that minimize the intracellular concentrations of the antibiotic as a result of poor penetration into the bacterium or of antibiotic efflux; second, those that modify the antibiotic target by genetic mutation or post-translational modification of the target; and third, those that inactivate the antibiotic by hydrolysis or modification (Blair et al. 2015).

Because of the two mechanisms of inherent resistance and develop resistance, the use of antibiotics will only aggravates the adaptive changes of gene and shape functions of the bacteria. As a result, there is an urgent need to develop alternatives to the role of antibiotics to address this serious problem, which is spreading around the world.

Antibiotics play a role in the treatment of infection by killing bacteria. However, as a kind of widely existing organisms, bacteria can also obtain the resistance of antibiotics through a variety of forms to avoid the risk of being killed (Cabello 2006). This resistance is called "bacterial resistance", and the bacteria that obtain drug resistance are called "drug-resistant bacteria".

Bacterial resistance is a natural phenomenon that is strengthened by human beings, and the transmission of drug-resistant bacteria is mainly divided into two ways: first, it can be produced by oral administration in the human body, and the phenomenon is called "self-producing". Secondly, after the administration of antibiotics to the livestock, these super-bacteria can also be transmitted to humans due to the contamination of the faecal urine or the residual problems in the agricultural and livestock products (Figure 2).
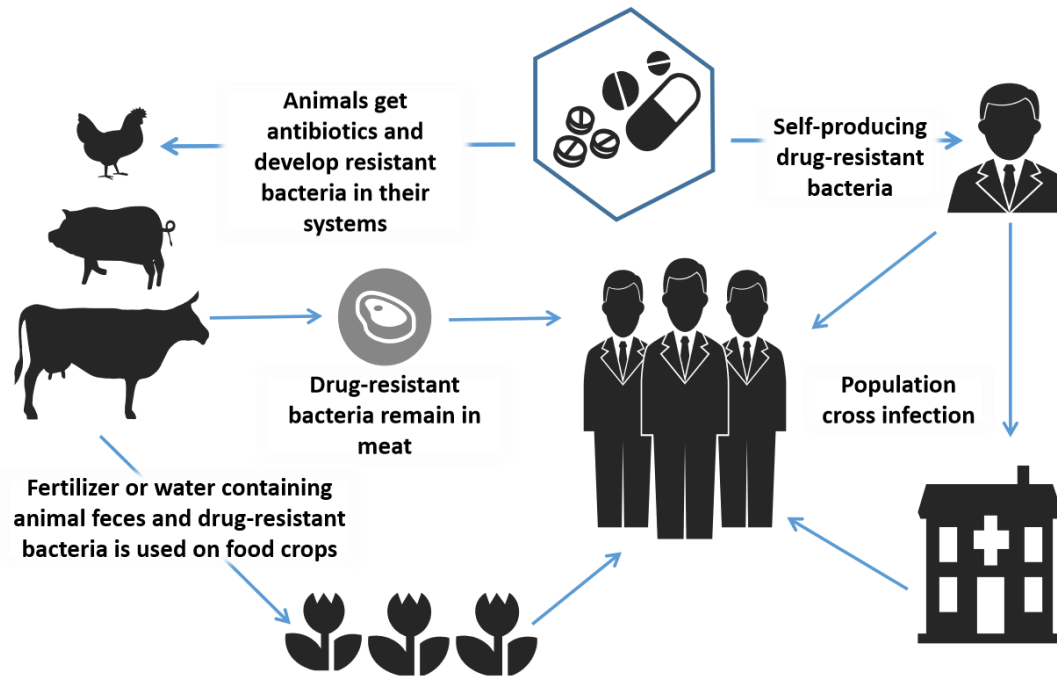
Figure 2. Mechanisms of antibiotic resistance dissemination.

2. Probiotics

1) The development history of probiotics

In 1953, German scientist Werner Kellas found that the fermentation of microorganisms was beneficial to the development of rumen in ruminants, and stressed that it was "an active substance necessary for the healthy development of life"(Warner 1953). As a result, the concept of probiotics began to form. In 1965, Lily and Steelwell named it 'a substance secreted by one organism and capable of stimulating the growth of another organism (Lilly and Stillwell 1965).' In 1992, Fuler defined probiotics as "a live microbial feed supplement that benefits host animals and benefits from improving their intestinal microbial balance (Gasbarrini, Bonvicini and Gramenzi 2016)." Since then, probiotics have been widely used in various aspects. Important events in the development history of probiotics can be referred to the Figure 3.

Bifidobacteria was discovered by Henry Tissier

'Probiotics' was first coined by D.M. Lilly& R.H. Stillwell

Probiotics were offically recognised by WHO and FAO as live micro-organisms

1890   1899   1907   1965   1995   2002   2013

Lactobacillus acidophilus was discovered by Ernst Moro

Consumption Bulgarian yogurt was good for health (Elie Metchnikoff)

'Prebiotics' was first coined by Marcel Roberfroid

'Psychobiotics' was conceived by Ted Dinan

Figure 3. History of probiotics.

Probiotics bring health benefits to the host mainly through three mechanisms (Figure 4). First of all, probiotics such as lactic acid bacteria can secrete lactic acid to inhibit the propagation of pathogenic microorganisms, or directly affect pathogens by producing bacteriocin(C. Prabhurajeshwar 2017) and other antibacterial substances. Secondly, probiotics can regulate the host immune system through non-specific immunity. Finally, probiotics can maintain the function of host intestinal barrier through fermentation (Oelschlaeger 2010). Probiotics are widely used in food and feed additives because of their stabilization of host intestinal flora.

2) Mechanism of probiotics

The intestinal microflora plays an important role in inhibiting the growth of gastrointestinal pathogens, maintaining the function of intestinal barrier and regulating immune homeostasis.

(1) Inhibition of pathogens

First of all, the interaction between intestinal microorganisms is very complex, in which the inhibition of intestinal pathogenic bacteria is essential to maintain the health of the host (Nagpal et al. 2012). Probiotics can inhibit the growth of pathogenic bacteria by producing antibacterial substances. Lactic acid

bacteria as a representative probiotic bacteria can produce lactic acid, bacterial

hormones, hydrogen peroxide and citric acid and other substances, these

substances have antibacterial effect. These inhibitors have been shown to inhibit

the growth of pathogens, including *E.coli*, Salmonella, Listeria, *Clostridium* and

Campylobacter (Gareau et al. 2010). Through these antimicrobial properties of

probiotics, they can protect hosts from a variety of pathogens and also can

function as alternatives to antibiotics.

(2) Gut barrier function

Second, probiotics can also maintain the function of intestinal epithelial

barrier and protect the tight junction between cells. Probiotics can secrete many

beneficial metabolites or molecules to regulate the integrity of tight intestinal

connections. Among them, the production of SCFA is one of the important

mechanisms to enhance the function of intestinal epithelial barrier (Radha

Krishna Rao 2013). The presence of microorganisms in the digestive tract can

stimulate the fermentation of intestinal substances, and make the intestinal tract

have high scfa production. These SCFA can regulate the high expression of tight

junction protein related genes in intestinal cells. In addition, lactic acid bacteria

can decompose glucose to produce lactic acid, thus regulating intestinal pH.

Increased levels of lactic acid in the intestine also contribute to the treatment of intestinal syndrome and dyspeptic diarrhea (Tsukahara and Ushida 2001).

(3) Immunomodulation

Third, some probiotic strains such as *L. casei subsp. rhamnosum, L. helveticus,* and *E. faecium* have been identified to have the property of increasing the non-specific immune response of host (Mack et al. 1999). Some of 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). However, due to the resistance of the mucus layer itself, most bacteria can not reach the epithelial cells smoothly. 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. 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, 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).
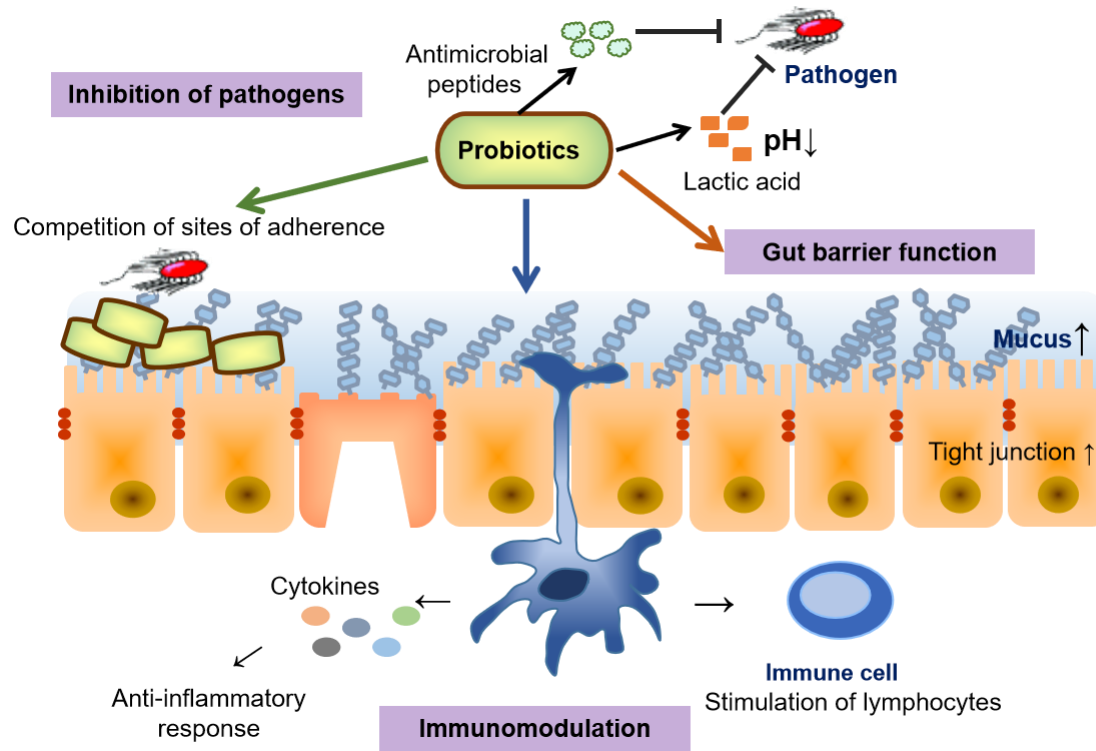
Figure 4. Biological effects and mechanism of probiotics.

Three kinds of mechanisms: resistance to pathogens, enhancement of intestinal barrier function and regulation of host immune

system.

3) Lactobacillus plantarum

(1) Lactobacillus plantarum **as probiotics**

Lactobacillus plantarum is a versatile lactic acid bacterium that is encountered in a range of environmental niches, has a proven ability to survive gastric transit, and can colonize the intestinal tract of human and other mammals (de Vries et al. 2006). The number of lactic acid bacteria in the intestines of humans and other animals may vary depending on the animal species, the age of the host, or the location in the intestines. Only a few *Lactobacillus* species contain representatives that are both involved in traditional and industrial food fermentations and reside in the mammal intestine which include *L.*crispatus, *L.*gasseri, and *L.*plantarum (Çataloluk and Gogebakan 2004).

(2) Genome shuffling for the evolution of different strains

An important objective of a biotechnological research is the engineering of microbial cells for the production of industrially valuable metabolites (K ATARZYNA L EJA 2011). The main objective of research into effective technologies is to improve bacteria strains that are able to produce metabolites, and which will find application in industries such as chemical, food,

pharmaceutical, and biofuel (Jixian Gong 2009). Currently, genome shuffling is one of the most efficient methods for the evolution of strains toward desirable phenotypes. Through this method, different genes which are associated with the production of metabolites can be recombined during several rounds of genome shuffling and consequently desirable phenotypes can be obtained (Jin et al. 2009). Genome shuffling is similar to the classical strain improvement in that it is a cycle of genomic diversification and screening for improved strains.

This technique can be simply understood as speeding up the evolution of bacteria. The process of genome shuffling mainly consists of two steps: the construction of parent library and the selection of phenotypes by protoplast fusion (K ATARZYNA L EJA 2011). The main process of genome shuffling method is showed in Figure 5.

First of all, in order to build the parent strain library, the original strain is designed to produce more genotypes. Then the required strains are collected to form a protoplast fusion parent library. Finally, protoplast fusion is carried out. One thing to note in this process is that if an inappropriate parent strain is selected, the required phenotype cannot be obtained . The next step in genome shuffling is the fusion of protoplasts. The cells are resuspended in a buffer

containing lysozyme or other enzymes (Rojan P.John, Dhanya Gangadharan and Nampoothir 2008), and then the protoplasts are collected by centrifugation (Otte et al. 2009). Finally, the required phenotypes were obtained from protoplast fusion populations by screening. Using this technique, the desired target strains can be screened out in a relatively short period of time.
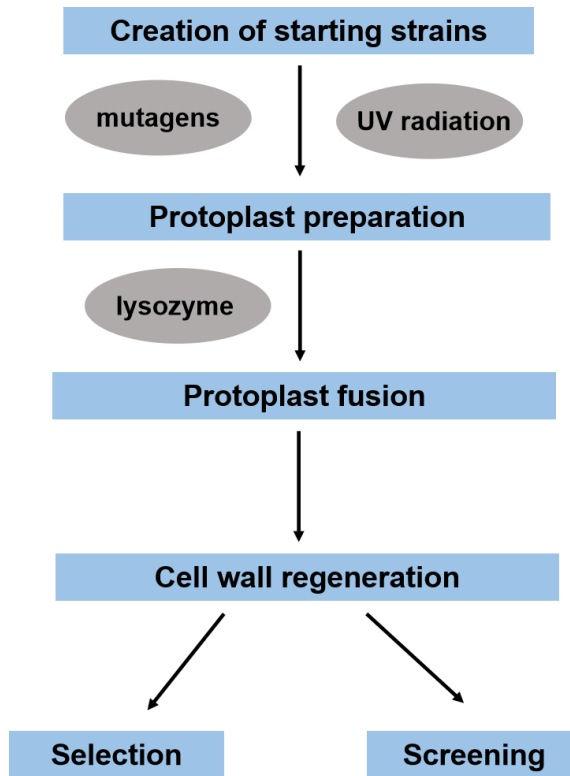
Figure 5. A general scheme of the genome shuffling process.
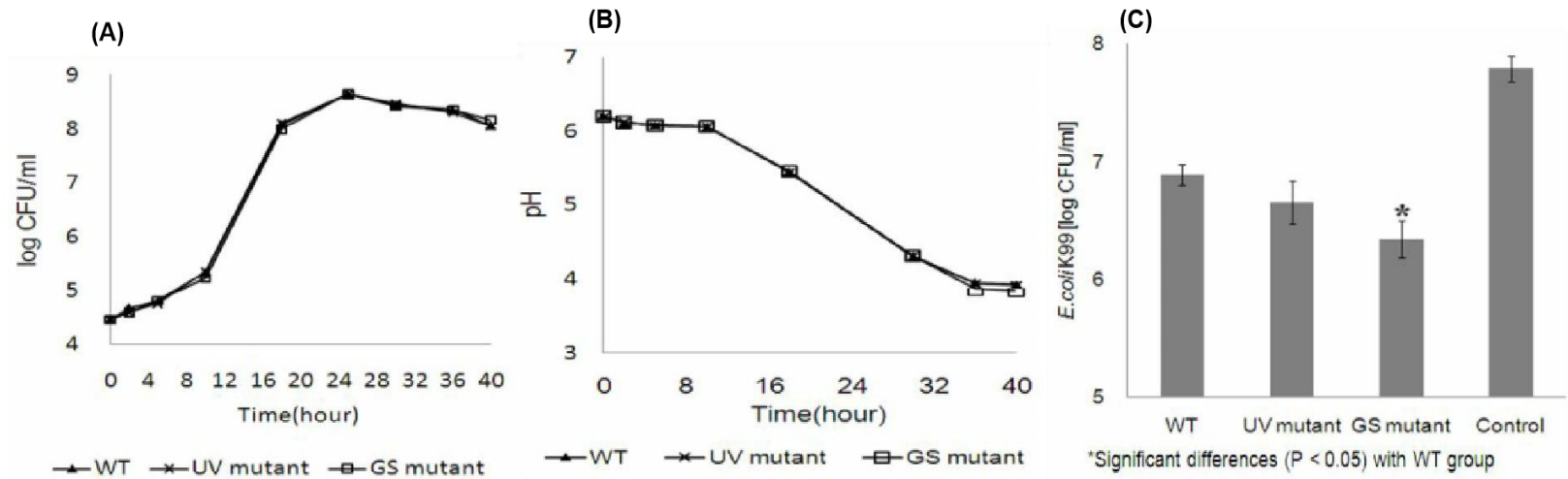
Figure 6. The growth characteristics and antibacterial ability of *Lactobacillus plantarum* WT, UV mutant and GS mutant.

(A) Growth curve, (B) pH curve and (C) antibacteial ability aganist *E.coli* K99 of *Lactobacillus plantarum* 177 (WT) and its

UV mutant and genome shuffling mutant (GS1). The experimental data was derived from (Seo 2012).

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(3) *Lactobacillus plantarum* **GS1**

To improve the antibacterial ability from the wild type strain, a mutant which named LP-GS1 (*Lactobacillus plantarum* genome shuffling mutant 1) was selected by genome shuffling technology (Seo 2012).

The growth characteristic of the strain was as shown in Figure 6, the UV mutant and the genome shuffling mutant did not change in the growth characteristic as compared with the wild type, but the antibacterial ability of the genome shuffling mutant to the *E.coli* K99 is obviously enhanced with respect to the wild type and the UV mutant by co-culture experiments.

3. Factors that affect the mass production of lactic acid bacteria

Fermentation with lactic acid bacteria (LAB) has long been used in the processing of different foods. Milk, meat and vegetable products, as well as silage, have been prepared using LAB starters in order to improve the flavour and texture of the product (Niku-Paavola et al. 1999). As a substitute for antibiotics, LAB has very good market prospects. However, due to the cost of

production and the limitation of cultivation technology, it is difficult to achieve economical mass production of lactic acid bacteria. Therefore, the development of large-scale production technology and subsequent freeze-drying technology is an important means for the production of probiotics-related industries for commercial use.

1) Medium composition

The major selection criteria that have been applied for probiotics include the ability to survive transit through the gastrointestinal tract, production of antimicrobial substances towards pathogens, a short generation time, a good shelf life in food or powdered préparations and so on (Goldin 2019). Therefore, it is very important to develop a mature production process for its efficiency.

MRS (deMan, Rogosa, and Sharp) medium introduced by de Man et al. has been widely used as a commercial medium for *Lactobacillus* species from various sources because it contains essential ingredients (polysorbate, acetate, magnesium, and manganese) as well as growth-inhibiting reagents to suppress the growth of unfavourable bacteria (de Man JD 1960).

However, many lactic acid bacteria still cannot be grown in the absence of crude materials that supply nutritive substances of undefined nature (McNuTT 1950). The content and effect of each component are shown in Table 1. Most lactic acid bacteria can only metabolize sugar and related fermented compounds to obtain energy, so they often grow where carbohydrates exist (Soyoung Yeo et al. 2018). Lactic acid bacteria generally have limited synthetic capacity, and they also need many other nutrients, including amino acids, vitamins, purine and pyrimidine (Hammes and Hertel 2006). It was found that lactic acid bacteria could propagate quickly in the medium containing peptone, yeast extract and beef extract. This is because peptone and beef extract provide nitrogen source for lactic acid bacteria, yeast extract provides amino acids, vitamins and inorganic salts for lactic acid bacteria (Aeschlimann and von Stockar 1990). Many studies have also proved that different lactic acid bacteria have specific requirements for nutritional elements (Soyoung Yeo et al. 2018). Therefore, in order to achieve industrial mass production, it is very important to choose the most suitable culture medium.

Table 1. The composition of culture medium.

Ingredient	Content % (w/v)	Role of different components
Glucose	2.0	Carbon source
Peptone	1.0	Nitrogen source
Beef extract	1.0	
Yeast extract	0.5	
Dipotassium hydrogen phosphate	0.2	Buffer
Sodium acetate trihydrate	0.5	Growth factor, inhibition of some miscellaneous bacteria
Manganese sulfate tetrahydrate	0.005	Growth factor
Tween 80	0.1	A surfactant which assists in nutrient uptake
Triammonium citrate	0.2	Growth factor, inhibition of some miscellaneous bacteria
Magnesium sulfate heptahydrate	0.02	Cations used in metabolism
Distilled water	94.475	Solvent

2) Culture conditions

The growth of lactic acid bacteria is influenced by various conditions, and pH is one of the factors. Lactic acid bacteria metabolize to produce lactic acid, which makes the initial neutral and alkaline medium pH gradually lower (TC Zhang 1996). However, the accumulation of lactic acid can also inhibit the growth of lactic acid bacteria, resulting in the decrease of OD value of lactic acid bacteria in the medium with good growth in the early stage (Coghetto et al. 2016). In the medium with initial pH of 7.0 and 7.5, the proliferation of lactic acid bacteria was significantly accelerated after 48 hours, and the OD value was the highest after 96 hours. At the same time, in the medium with initial pH of 3.0-6.5, OD increased first (0-96 h) and then decreased (96-120 h). This may be related to the decrease of medium pH during bacterial culture (Guarner et al. 2007).

In addition, other factors, such as temperature and culture time, also affect the number of bacteria by affecting the activity of metabolic enzymes of lactic acid bacteria (Coghetto et al. 2016).

3) Freeze-drying process

Freeze-drying is a drying method that freezes the water in the material into ice and then sublimates the ice to remove the water. Because lactic acid bacteria are very sensitive to heat (Soyoung Yeo et al. 2018), one of the best ways to make products with high stability after fermentation is to freeze-dry them. Compared with other methods, freeze-dried products can not only ensure a higher survival rate, but also quickly and easily dissolve and easy to use.

Freeze-drying is also a complex phase transformation process. The various stresses in the whole process of freeze-drying products, including low temperature stress, freezing stress and drying stress, are often the direct or indirect factors leading to protein denaturation in products. Therefore, protective agents should be used in the process of freeze-drying. In order to obtain ideal lyophilized products, suitable cryoprotectants are very important (Reddy et al. 2009).

(1) Lyophilization and cell damage

During the freeze-drying process, the sample is gradually solidified at low temperature. At this stage, all fluids exist in solid, crystalline or glass states (Siaterlis, Deepika and Charalampopoulos 2009). Usually, water becomes a

complex ice crystal network structure, but it is also compacted back to the glass structure or somewhat retained with a gap structure. The solute is concentrated and may eventually crystallization and precipitate (Reddy et al. 2009). At the same time, the volume expansion of the system may produce very strong mechanical pressure, and the increasing concentration of fluid in the gap will also produce osmotic pressure impact (Figure 7).

The cell damage caused by freezing is mainly classified into two hypotheses: The first on is mechanical damage which is caused by intracellular and extracellular ice formation. And the second one is called solution effect damage which is caused by osmotic pressure and protein denaturation (Siaterlis et al. 2009).

It is generally believed that mechanical damage is the main cause of cell damage. When the cell is frozen rapidly, the water inside the cell is frozen before it can infiltrate, and the mechanical force produced by the growth of the ice crystal will cause cell damage (Zhang et al. 2017). When the cell is rehydrated, the intracellular ice crystal will be recrystallized, and there will be enough power to destroy the plasma membrane or the cellular organs of the

membrane, such as mitochondria; at the same time, it will also destroy the protein colloid which leads to the death of cells.

There are two kinds of cell damage caused by solute effect:

① With the increase of ice crystals in the external environment, the extracellular osmotic pressure increases gradually. When the intracellular water spills, that is, when the cell shrinkage reaches the critical minimum volume, the permeability of the cell membrane will be increased irreversibly and fatally, and the solution that could not penetrate the membrane will become permeable, resulting in cell death (Bhattacharya and Prajapati 2016).

② Due to the destruction of cell membrane structure caused by lipid peroxide caused by freezing injury, the permeability of cell membrane will be greatly improved. Intracellular proteins are very sensitive to electrolytes, especially in the presence of high concentrations of electrolytes (Yang et al. 2016), which can cause protein denaturation, loss of function and increase the possibility of cell death. In addition, with the increase of solute concentration, the intracellular pH value and ion content will change (Yeo et al. 2018), and the incidence of

harmful chemical reactions will also increase (Reddy et al. 2009). The higher

the concentration of the gap liquid, the more severe the cell damage.
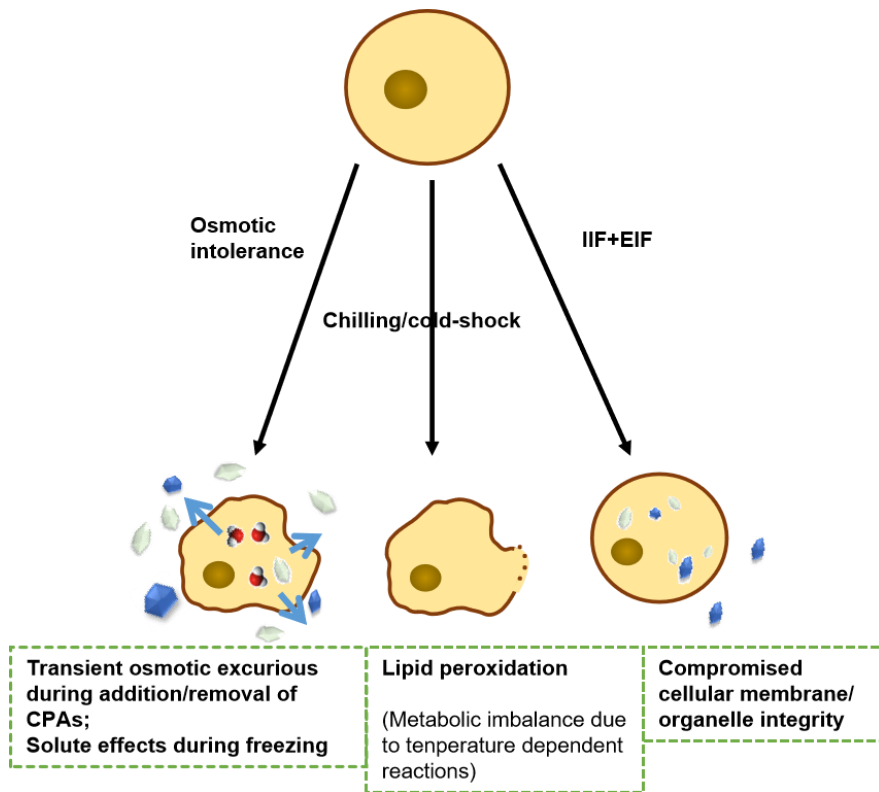
Figure 7. Potential mechanisms of damage that can occur during freeze-drying process.

(2) Cryoprotectant and its protective mechanism

Protective agents can change the physical and chemical environment of biological samples during freeze-drying, reduce or prevent the damage of cells caused by freeze-drying or rehydration. Maintaining the original physiological and biochemical characteristics and biological activities as far as possible (Reddy et al. 2009). Freeze-drying protectants can not only affect cell survival during freeze-drying, but also affect the stability of proteins and cells during storage (Siaterlis et al. 2009).

Freeze-dried protective agents have different classification methods. According to their molecular weight and whether they can penetrate into cells (Bhattacharya and Prajapati 2016), there are two types: permeable protective agent and non-permeable protective agent. Depending on whether they penetrate the cell wall or membrane, they can be subdivided into three types: (1) protectants that can penetrate both cell wall and cell membrane (e.g. glycerol); (2) protectants that can penetrate the cell wall but cannot penetrate the cell membrane (e.g. monosaccharides and partial disaccharides). (3) Protective

agents (such as trehalose, polysaccharides, polyethylene glycol, etc.) that do not penetrate cell walls and membranes (Reddy et al. 2009).

Penetrating cryoprotectants can pass through the cell membrane and bind to intracellular water molecules. By hydration, the viscosity of the solution is increased, and the crystallization process of water is weakened, thus can protect cells from the freezing process (Yang et al. 2016). The concentration of various protectants, the ability of penetrating into cells, and the effect on the activity of water molecules are different.

Non-penetrating cryoprotectants cannot enter the cells because of their large molecular weight. It will make the solution undercooled and reduce the solute concentration at a specific temperature, thus playing a protective role. According to its chemical properties, protectants can be divided into polyhydroxyl compounds, carbohydrates, amino acids, polymers, proteins and so on (Reddy et al. 2009).
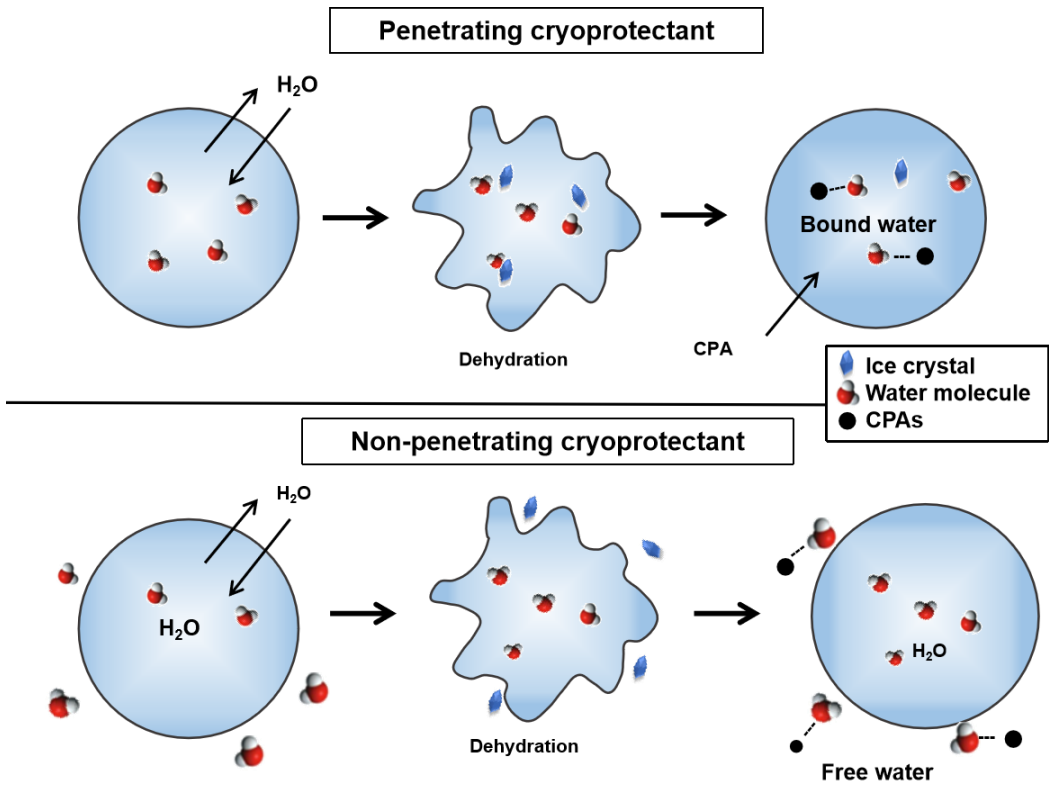
Figure 8. Schematic representation of the manner by which penetrating and non-penetrating CPAs exert their actions upon cells.

4. Mass production strategy

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in analytical chemistry as a means of discovering conditions (Pedro W. Araujo 1998) at which to apply a procedure that produces the best possible response (Bezerra et al. 2008). In order to realize mass production, it is necessary for the optimization process of production conditions.

The "one factor at a time" is the most commonly used operation in the optimization of experimental conditions. The main way is to keep the level of other factors constant, and to optimize the product level after treatment with different concentrations of a target factor. This optimization technique is massive, time consuming and usually ignores the importance of interactions between various physical and chemical parameters (Malheiros et al. 2015).

In general, when optimizing a production process, if the factors involved in the analysis are independent, the most common practice is to experiment with one factor at a time (OFAT) while keeping all other factors unchanged.

However, because the interaction of various conditions in the reaction system is not taken into account, the results are often misleading and fail to reproduce conclusions drawn from such an exercise. There is now increasing recognition that this traditional experimental approach ought to be replaced by soundly based chemometric methods (Stalikas et al. 2009). In order to solve these problems, a more effective method is to study the interaction of various factors at the same time through the design of experimental statistical technology to screen the really important factors in the target production mode and to determine the optimal level combination.

Response surface methodology (RSM) was developed by Box and Wilson (1951) and since then it has been widely used as a technique for designing experiments. The RSM method is based on the fit of mathematical models (linear, square polynomial functions and others) to the experimental results generated from the designed experiment and the verification of the model obtained by means of statistical techniques. As a powerful tool in designing the experiments and optimizing different environmental processes (Karimifard and Alavi Moghaddam 2018), response surface methodology (RSM) is basically a collection of mathematical and statistical methods (Behbahani,

Moghaddam and Arami 2011) that is useful for designing the experiments, developing models by considering the interactions of parameters, and process optimization (Khedmati, Khodaii and Haghshenas 2017, Haghshenas et al. 2015).

Plackett-Burman design method mainly analyzes the two levels of each factor, and determines the significance of the factor by comparing the difference between the two levels of each factor and the difference of the whole factor (Jacques et al. 1999). The design of screening test cannot distinguish the main effect from the influence of interaction, but the factors that affect the significant effect can be determined (Ahuja, Ferreira and Moreira 2004), so as to achieve the purpose of screening and avoid wasting the test resources because of too many factors or some of the factors are not significant in the later optimization experiment (Wasko et al. 2010).

Central Composite Design has been widely used in the application of response surface methodology (M.Ahmadi et al. 2005). The second order response surface model is fitted by selecting corner, axis and center points. Because CCD requires a relatively large number of sample points, it can only

be selected when the total number of important variables is reduced to an acceptable number in the late stages of RSM application (A.R.Khataee, M.Fathinia and S.AberM.Zarei 2010) . And also because of the relatively large number of sample points, the CD experimental model has a high prediction accuracy for the real results.

The main objective of RSM is to obtain the optimum operational conditions for the system or to acquire a region that satisfies the operating specifications (Zhang, Zeng and Cheng 2016). The parameter optimization process of the system consists of the following five basic steps:

(1) selection of independent variables and possible responses, (2) selection of experimental design strategy, (3) execution of experiments and obtaining results, (4) fitting the model equation to experimental data and obtaining response graphs and verification of the model (ANOVA), and (5) determination of optimal conditions (Witek-Krowiak et al. 2014).

The specific steps are shown Figure 9.
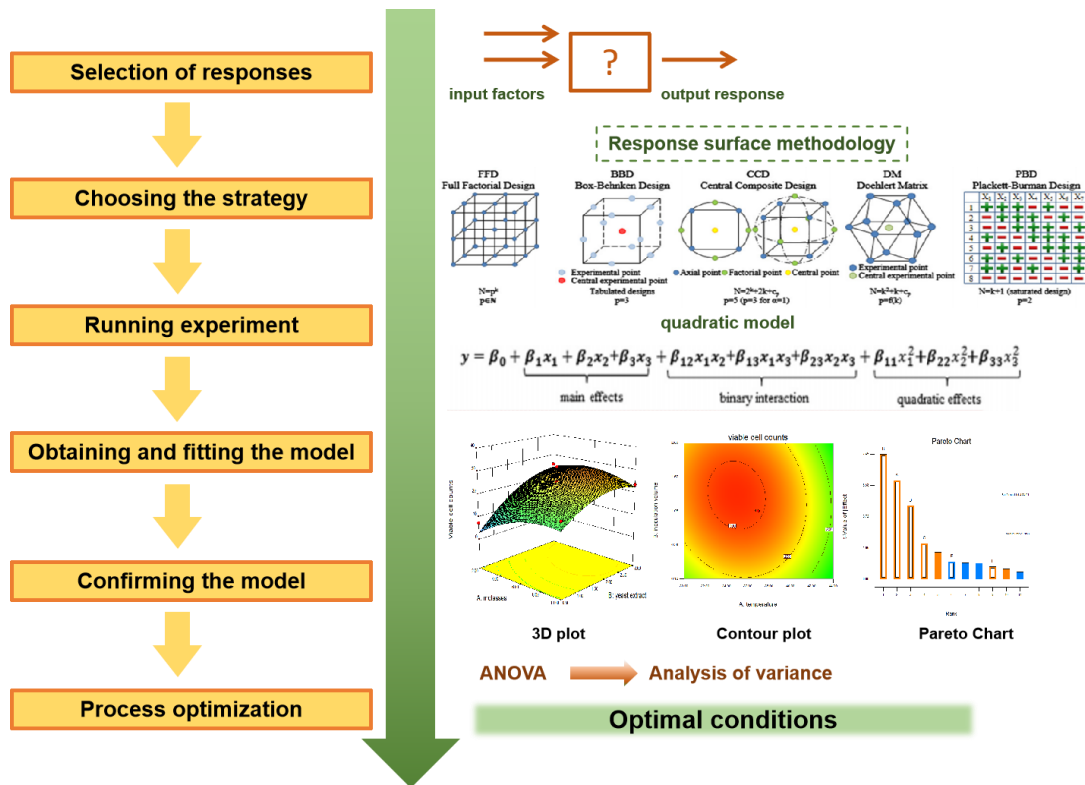
Figure 9. Design of experiment in RSM methodology.

1) Screening of variables

Many variables will have impacts on the corresponding system studied. In order to determine the influence of different factors on the required response value, it is necessary to select the variables with great influence. The screening design should determine which experimental variables and their interactions have more significant effects. Full or fractional two-level factorial designs may be used for this objective principally because they are efficient and economical (Bezerra et al. 2008).

2) Choice of the experimental design

Among the more known second-order symmetrical designs are the three-level factorial design, Box–Behnken design, central composite design, and Doehlert design. These symmetrical designs differ from one another with respect to their selection of experimental points, number of levels for variables, and number of runs and blocks (Bezerra et al. 2008). In the actual experiment, it is necessary to make an appropriate choice according to the situation.

3) Running the experiment

Converts the actual response value of each study to an encoding with dimensionless values, which must be proportion alatits localization in the experimental space (Boehm et al. 1997). Codification is of concern because it enables the investigation of variables of different orders of magnitude without the greater influencing the evaluation of the lesser (Puri, Beg and Gupta 2002).

4) Evaluation of the fitted model

The main principle of ANOVA is to compare the changes caused by processing and the changes caused by the inherent random errors in the measurement of the response. From this comparison, the source of experimental variance can be taken into account to evaluate the significance of predictive response.

5) Determination of the optimal conditions

After the significance of the model is determined by statistical analysis, the linear surface reflected by the model can be used to predict the optimal result (the corresponding value of the highest point of the surface) in order to achieve

the optimization of the conditions (Bezerra et al. 2008). However, in order to ensure the feasibility of the conditions, it is necessary to carry out practical experiments to verify the prediction results (Witek-Krowiak et al. 2014).

Many applications of the central composite design in the optimization of analytical procedures can be found in the literature (Karimifard and Alavi Moghaddam 2018). Therefore, it is a suitable technology for lactic acid bacteria culture optimization.

Study 1. Optimization of large-scale production process of *Lactobacillus plantarum* GS1

1. Introduction

With the emergence of antibiotic resistance, antibiotic abuse has caused serious biological and ecological problems worldwide. Probiotics, known as beneficial microbes, are being proposed as an effective and eco-friendly alternative to antibiotics. The beneficial actions probiotic bacteria provide include aid in lactose digestion, resistance to enteric pathogens, anti-colon cancer effect, small bowel bacterial overgrowth, prevent allergies, and immune system modulation (Sanders and in't Veld 1999).

Lactic acid bacteria (LAB) have been used as food supplements and are highly valued for their probiotic properties. Many probiotic isolates, such as *Lactobacillus plantarum* Lp01, *Lactobacillus acidophilus* LA1, and *Lactobacillus rhamnosus* GG, are already widely used and produced on an industrial scale (Bhatt and Srivastava 2008). Other studies have shown that *L.acidophilus* can be used as a probiotic or living organism, which upon

ingestion in certain numbers (10^7-10^9 cfu/day), exert health benefits beyond inherent basic nutrition.

However, because LAB are fastidious and strain-dependent with respect to nutrient and environmental requirements, a rich medium and suitable conditions are required for good growth of each strain (Ahmad et al. 2013). In addition, the freeze-drying and subsequent storage lead to a decrease in cell viability because the drying process exposes the cells to an additional stage of stress processing. These factors may cause osmotic shock and membrane injury during recrystallization by the formation of intracellular crystals. Therefore, the industrial application of LAB depends on the concentration and conservation technologies that are required to ensure the long term stability of cultures in terms of viability and functional activity (LI et al. 2010).

RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables, and the objective is to optimize this response. RSM has important applications in industrial fields for new production process design, development and improvement of existing product design. Many studies

have reported the use of RSM to achieve mass production of lactic acid bacteria. Toward this objective, linear or square polynomial functions are used to describe the system studied and consequently, to explore (modeling and displacing) the experimental conditions until its optimization.

Study 1 (Figure 11) was to optimize the mass production process of LP-GS1 by OFAT (one factor at a time), PBD (Plackett-Burman design) and CCD (Central composite design) analytic procedures (Figure 10) on the premise of maintaining the original excellent antibacterial ability. In study 1-1, the composition of the carbon and nitrogen sources of the culture medium and the culture conditions were improved so that a high density culture was achieved at a relatively low cost. In study 1-2, the high survival rate and long storage stability were achieved by optimizing the freeze-drying process. This study laid a foundation for the commercialization of LP-GS1.
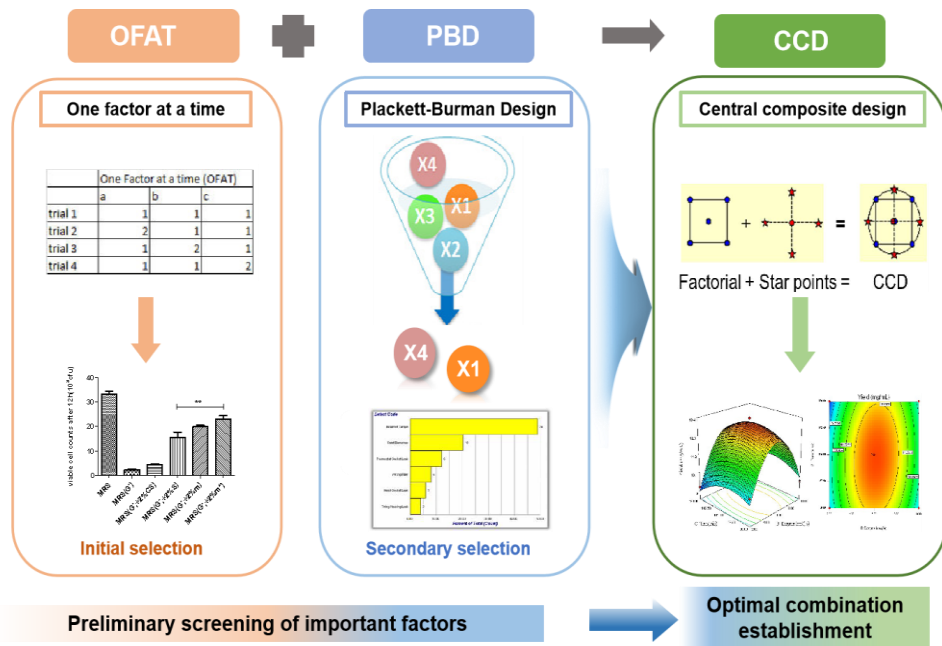
Figure 10. Statistical analysis technique used in study 1.

At the beginning of the experiment, the traditional OFAT and PBD experimental analysis methods were used to select a variety of production factors. After that, to optimize these selected factors and to consider the influence of their interactions on the final response, the CCD analysis method was used to establish a model, and the optimal response conditions were predicted based on the function of the model.
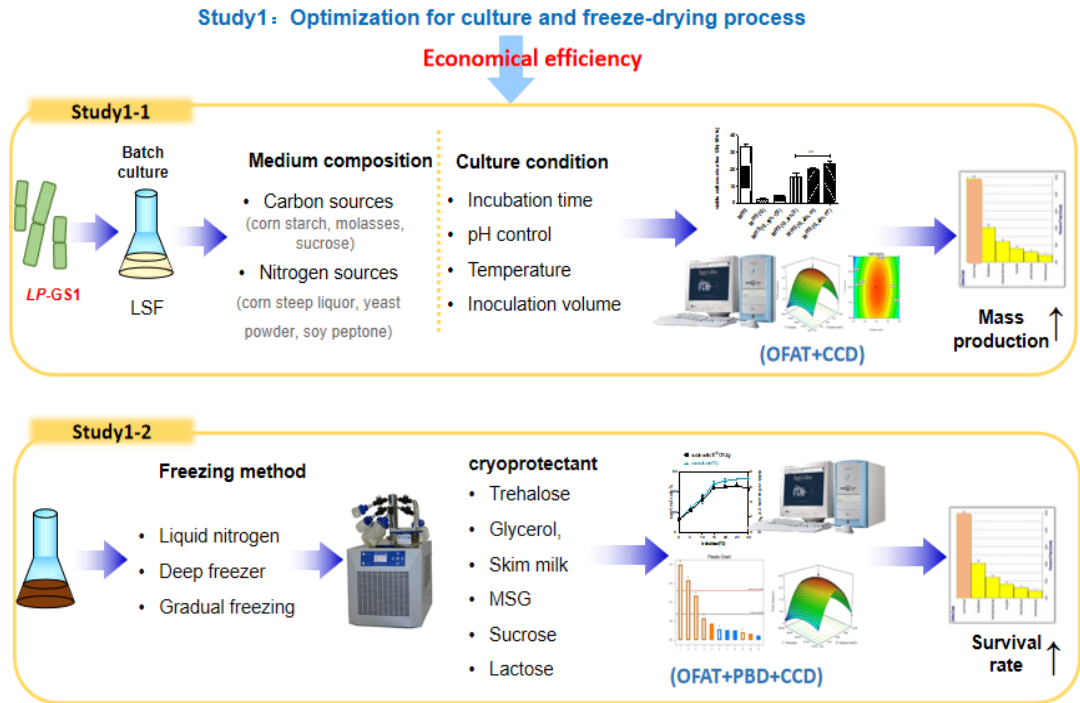
Figure 11. Schematic diagram of study 1.

The aim of study 1 was to optimize the medium compositions, culture conditions and freeze-drying processes of LP-GS1 by OFAT (one factor at a time), PBD (Plackett-Burman design) and CCD (Central composite design) methods on the premise of maintaining the original excellent antibacterial ability.

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 and MacConkey agar were purchased from BD Difco (Sparks, MD, USA) for bacterial cultures.

2) Strain preparation

L.plantarum 177 and *L.plantarum* GS1 were stored at -70℃ in a glycerol based freezing medium (15% glycerol salt solution; 75% MRS culture medium) for long term storage.

For seed culture, streaking was performed on MRS agar plate using the bacteria stock which is stored in -70℃ deep-freezer. After culture at 37℃ for 24 hours, one colony was selected and inoculated in 10ml MRS broth and cultured with overnight to increase the activity of bacteria.

3) Co-culture for antimicrobial activity

The antimicrobial activities of LP 177 and LP-GS1 against *E.coli* K99 were determined using the co-cultivation assay (Ditu et al. 2011) with some modifications (Figure 12). To quantitatively compare the antimicrobial activity of *L.* plantarum against *E.coli* K99, 2.0 x 10^7 CFU/ml of *E.coli* was co-cultured with 2.0 x 10^6 and 2.0 x 10^5 CFU/ml of LP in MRS broth for 12h at 37 °C with aerobic condition in a shaking incubator (200 rpm). The antibacterial activity of LP177 and LP-GS1 against *E. coli* K99 during co-culture can be compared directly by the survival rate of *E. coli* K99. After culture, the samples were diluted with appropriate multiples and spread on MacConkey agar, incubated for 12 h at 37 °C and the number of *E. coli* K99 colonies was counted.
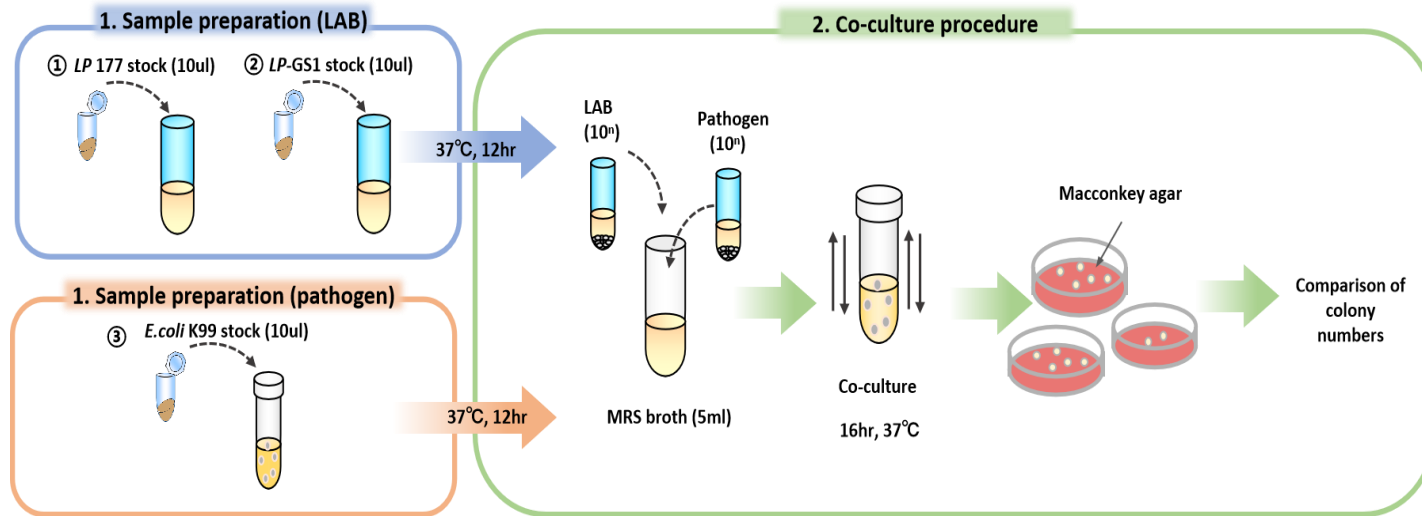
Figure 12. Procedure of measuring antimicrobial activity of LP 177 and LP-GS1.

Quantitative analysis for measuring antimicrobial activity of LP against pathogens. Viable cell counts of pathogen was measured

using MacConkey agar.

4) Preparation of cryoprotectant

All the candidate cryoprotectants were first formulated with three distilled water to form a stock solution. Among them, skim milk (BD Difco, Sparks, MD, USA) and glycerol were sterilized at 110 ℃ for 10 minutes. Trehalose, MSG, lactose and sucrose were sterilized with 0.22 μm filter membrane.

5) Vacuum freeze-drying process

The bacteria culture solution was centrifuged at 4℃, 6000 rpm for 15 minutes and the supernatant was discarded to obtain the cultured cells. Then cells were suspended in 1×PBS at the ratio of 1:10 (w/w) and washing under the same centrifugal conditions for 3 times. Finally, cells were mixed with different cryoprotectants in the proportion of 1:1 (w/w). Then, after pre-freezing in liquid nitrogen or in the refrigerator, samples were desiccated in a vacuum freeze-dryer at a condenser temperature of − 80°C.

6) Calculation of survival rate

After freeze-drying, the samples were weighted and then resuspended with 1×PBS. After gradient dilution, take 100μl of diluent which was in the appropriate concentration and spread it evenly on the MRS plate. After culture at 37℃ for 24 hours, living bacteria were counted. The survival rate was calculated by the formula:

$$\text{Survival rate}\,(\%) = \frac{\text{viable cells after freeze-drying}\,(\text{CFU})}{\text{viable cells before freeze-drying}\,(\text{CFU})} \times 100\%$$

7) Central composite design

For medium optimization, a two-factor, five-level CCD with 13 runs was employed. The tested variables (molasses and yeast extract) were encoded as A and B, and each variable was evaluated at five different concentration levels: combining factorial points (−1, +1), axial points (−α, +α), and central point (0), as shown in Table 2. The values of -1 and +1 were determined on the basis of the preliminary experimental results of OFAT.

For the optimization of culture conditions, a four-factor, five-level CCD with 30 runs was employed. The tested variables (temperature, inoculation volume,

incubation time and initial pH) were encoded as A, B, C and D, respectively. Each variable was evaluated at five different concentration levels as shown in Table 6. For the optimization of freeze-drying process, a three-factor, five-level CCD with 20 runs was employed. The tested variables (skim milk, trehalose and MSG) were encoded as A, B, C, respectively. Each variable was evaluated at five different concentration levels as shown in Table 13.

After the experiment was completed, the Y set in each experiment was used as the dependent variable or reaction, and then second-order model used to fit the response to the independent variables is shown as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j$$

Here, Y is the predicted response (output factors), X_i and X_j are input variables that affect response Y. k is the number of variables, β_0 is a constant term, and β_i is the ith linear coefficient. β_{ii} is the first quadratic coefficient and β_{ij} is the ijth interaction coefficient (Zhou et al. 2011).

8) Plackett-Burman design

In order to determine which of the six alternate cryoprotectants (skim milk, trehalose, glycerol, MSG, sucrose, lactose) had significant protective effect in the process of freeze-drying, PBD design was adopted. First of all, 11 factors (including 6 variables and 5 virtual variables) were evaluated by using 13 rounds of Plackett-Burman design. Because a center point was added, each variable was checked at three levels: −1 for the low level, +1 for the high leve and 0 is intermediate. Table 10 lists the variables used in the experimental design and their corresponding levels. The values of -1 and +1 were determined on the basis of the preliminary experimental results of OFAT. The response values of Plackett-Burman design and cell survival rate are shown in Table 11.

9) Model validation and confirmation

According to the prediction conditions given by RSM, the feasibility of the model is verified by practical experiment. The experiment was carried out in a 100 ml Erlenmeyer flask and the yield of LP-GS1 was obtained by viable cell count. The percentage deviation between prediction and experimental values was studied. All experiments were performed in triplicates and the average of the three independent experiments was taken as the result.

10) Statistical analysis

Variance analysis (ANOVA) was used to judge the significance between the model and the regression coefficient. The discriminant coefficient R^2 was used to judge the quality of the polynomial equation, and Fischer's *F*-test was used to test its statistical significance. The 3D response surface and contours plots predicted by the model were used to evaluate the interaction between input factors. Design-Expert Version 8.0.6.1 (Stat-Ease Inc., Minneapolis, MN, USA) was used for all of the designing experiments as well as for regression and graphical analysis of the experimental data obtained.

3. Results

1) Optimization of medium compositions

(1) Selection of important factors in culture medium

In order to confirm the effect of various components in commercial medium (MRS broth) on the number of LP-GS1, the experimental method of OFAT was used in the initial stage of the experiment. After classifying the original components in the culture medium, LP-GS1 was cultured under the condition of removing one of them.

The result showed that among the 10 components existing in MRS medium, carbon source and nitrogen source, which were used as nutrients for cell growth, had obvious effects on the number of bacteria (Figure 13). Although the number of cell counts in other groups also decreased compared with the control group, there were no statistical differences. Therefore, it can be determined that the selection of carbon source and nitrogen source is the most critical factor in order to realize the mass production of LP-GS1.
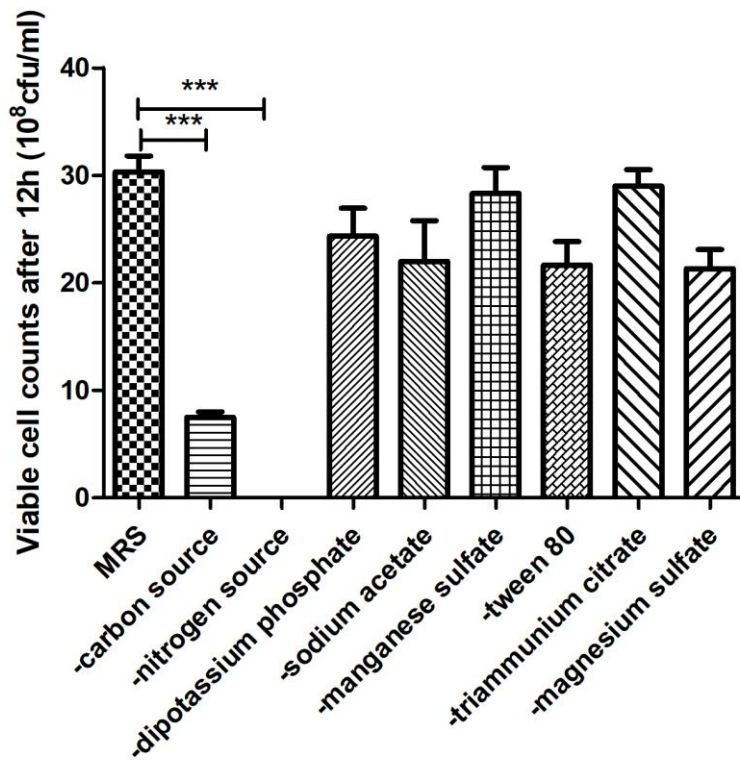
Figure 13. Selection of important factors in MRS broth.

After 12hrs of culture in medium with different components deficiency, the number of living bacteria was detected by MRS agar.

(2) Selection of carbon source and nitrogen source with OFAT method

In order to find a suitable alternative carbon source, various carbon source candidate components were added to the growth medium without the original carbon source (glucose). The number of bacteria in the culture medium was determined after culture under the same conditions.

The results showed that molasses not only had relatively low price, but also had the best microbial effect among all the carbon sources (Figure 14A). As a result, molasses were selected as an alternative carbon source. In order to determine the optimum addition amount of molasses, the concentration gradient experiment was carried out. The results showed that the highest level of viable cell counts could be obtained when the addition amount of molasses was 8% (Figure 14B). When the level of molasses added continued to increase, the number of bacteria in the medium decreased, which was thought to be due to the adverse effect of too high extracellular osmotic pressure on the growth of bacteria.
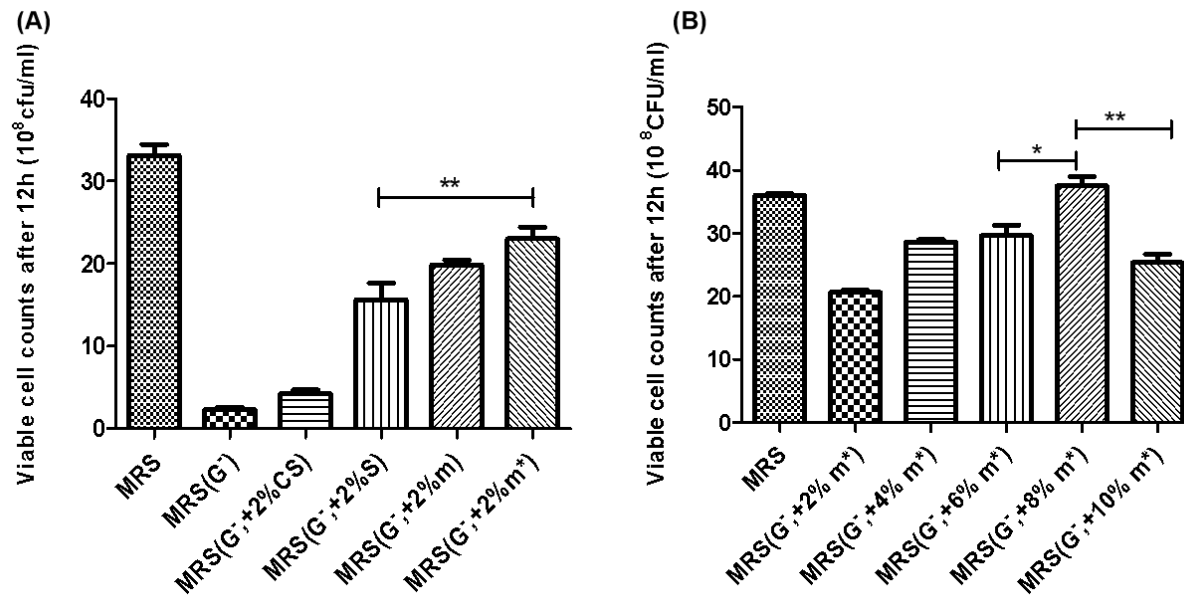
Figure 14. Effects of different carbon sources on the number of bacteria. (A) Effects of different carbon sources on the number of bacteria growing. (B) Effect of different concentration of molasses on the number of LP-GS1.

G^-: glucose free, CS: corn starch, S: sucrose, m: molasses, m*: molasses after centrifugation.

After determining the type and dosage of carbon source, in order to select the alternative nitrogen sources, the original carbon source (glucose) and nitrogen sources (peptone, beef extract, yeast extract) were removed from MRS broth. 8% of molasses was added as carbon source and 1% of the different nitrogen source candidate substance was added to the different treatment groups.

After culture with a variety of nitrogen sources, the number of viable cells in the treatment group which was treated with yeast extract had the closest viable cell numbers with control group (Figure 15). Therefore, yeast extract was the most suitable alternative nitrogen source for mass production of LP-GS1. The results of concentration gradient test showed that the best microbial effect was achieved when the addition amount of yeast extract was about 2% (Figure 15B).
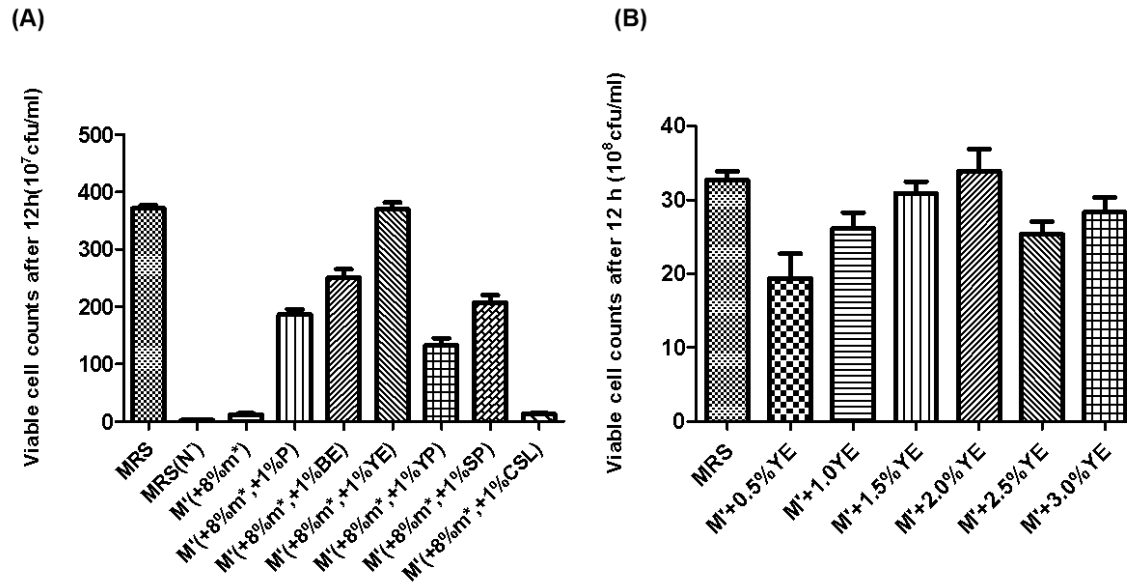
Figure 15. Effects of different nitrogen sources on the number of bacteria. (A) Effects of different nitrogen sources on the number of bacteria growing. (B) Effect of different concentration of yeast extract on the number of LP-GS1.

N⁻: nitrogen free, M': MRS (carbon and nitrogen free), P: peptone, BE: beef extract, YE: yeast extract, YP: yeast powder, SP: soy peptone, CSL: corn steep liquor, m*: molasses after centrifugation.

(3) Prediction of optimum ratio of carbon and nitrogen source by RSM

The next stage CCD was employed to study the interactions between the two significant factors (molasses and yeast extract) and also to calculate their optimal ratio. According to the results of OFAT experiments, the ranges of the two variables were decided as 2%-10% (20–100g/L) for molasses, and 0.5%-3% (5–30g/L) for yeast extract (Table 2). The experimental design and the results are presented in Table 3.

Table 2. Experimental range and levels of the independent variables for CCD design.

Independent variables	Range and levels				
	$-\alpha$	-1	0	1	$+\alpha$
A-molasses	0.34	2.00	6	10.00	11.66
B-Yeast extract	0	0.50	1.75	3.00	3.52

Table 3. Experimental design matrix using RSM with CCD and responses for cell counts and price.

Run No.	Variables		Response	
	Molasses	Yeast extract	Viable cell counts	Price
	%	%	$\times 10^8$ CFU/ml	$/L
1	6.00	1.75	25.3333333	0.079
2	10.00	0.50	18.2933333	0.044
3	10.00	3.00	23.4133333	0.135
4	6.00	3.52	24.9066666	0.1432
5	11.66	1.75	17.6533333	0.0939
6	2.00	3.00	25.6533333	0.1139
7	6.00	0	0.10666666	0.016
8	6.00	1.75	31.6266666	0.079
9	0.34	1.75	10.1333333	0.064
10	6.00	1.75	25.12	0.079
11	6.00	1.75	22.6666666	0.079
12	6.00	1.75	20.8533333	0.079
13	2.00	0.50	7.30666666	0.023

According to the treatment combination described (Table 3), the viable cell counts and the production price in this study were optimized. By fitting the experimental responses with the least squares method, two second-order polynomial regression models were obtained as the linear model and the quadratic regression model for the response surface simplification.

The experimental results of the CCD were fitted with a second-order polynomial equation for estimation of biomass concentration:

$$Y_1 = +25.10 + 2.42 \times A + 7.36 \times B - 3.31 \times AB - 4.22 \times A^2 - 4.97 \times B^2 \tag{1}$$

Here Y_1 is the predicted response of the bacterial viable cell counts ($\times 10^8$ cfu/ml), A and B are the actual values of molasses (%, w/v) and yeast extract (%, w/v), respectively.

Table 4. ANOVA analyse result.

	F-value	*p*-value	Sig
Model	7.43	0.0101	**
A-molasses	2.24	0.1780	NS
B-yeast extract	20.60	0.0027	***

***$P< 0.01$, **$P< 0.05$, *$P< 0.1$.
$R^2 = 0.8414$, Pred-$R^2 = 0.2750$, Adj-$R^2 = 0.7281$.

The results of ANOVA (Table 4) showed that "F-value" of the model was 7.43, and the "p-value" was < 0.05, which indicates that the model is significant. Similarly, yeast extract also had a significant effect on the final bacterial concentration. However, molasses did not significantly affect cell density in the designed variable level range. This may be due to the failure of statistical effect caused by too small selection of experimental interval.

The response surface diagram and the corresponding contour diagram are based on molasses (A) and yeast extract (B), and the interaction between the two independent variables was shown in Figure 16. Y value increased gradually with the increase of the concentration of molasses and yeast extract, then Y value decreased gradually with the further increase of the two factors after reaching the peak.

The coefficient of determination (R^2) was calculated as 0.8414 for viable cell count which indicated good agreement between the experimental and the predicted values. And the degree of discretization between the predicted value and the actual value was visualized by Figure 17.

(A)

(B)
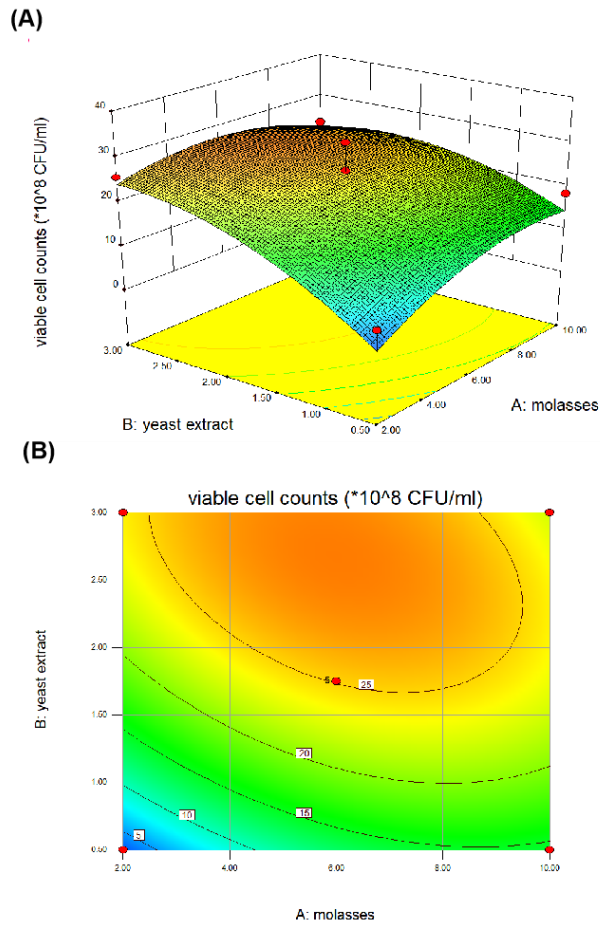
Figure 16. Three-dimensional response surface plots and two-dimensional contour plots for viable cell counts. (A) Three-dimensional response surface plots for viable cell counts showing variable interactions of molasses and yeast extract. (B) Contour plot of the model equation fitted to the data of the central composite design experiment.
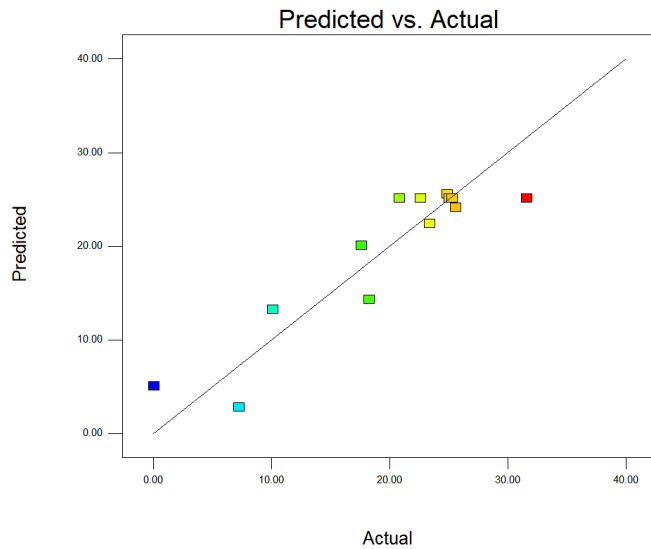
Figure 17. Predicted and actual experimental value of optimal culture medium compositions predicted by RSM.

Table 5. RSM prediction results for medium composition.

	Goal	Lower limit	Upper limit	
A- molasses	Is in range	2	10	
B- yeast extract	Is in range	0.5	3	
Viable cell counts	Maximize	0.1067	31.6267	
Price	Minimize	0.016	0.1432	
Solution				
Molasses	Yeast extract	Viable cell counts	Price $/L	Desirability
6.72	1.21	2.15×10^9 CFU/ml	0.0613	0.661

According to the results of statistical design, the optimum conditions were molasses 6.72%, yeast extract 1.21%, and the viable cell count was predicted to be 2.15×10^9 cfu/ml under this culture condition.

The theoretical values obtained by this method were tested in practice. The results showed that the actual number of bacteria reached 2.33×10^9 cfu/ml after being cultured in the optimized medium. Because there was no significant difference between the actual value and the theoretical value, the predicted results of RSM can be judged to be reliable, and the carbon source and nitrogen source composition of *LP*-GS1 medium was finally determined.

2) Optimization of culture conditions

After determining the composition of the medium, in order to further increase the yield, CCD analysis was conducted to optimize the conditions for cell culture (temperature, °C), inoculation ratio (v/v %), incubation time (hour), and initial pH (initial pH), which should be considered when incubating *Lactobacillus spp*. The ranges of the four variables were decided as 30-44℃ for temperature, 0.1%-2% for inoculation volume, 12-21 hrs for incubation time and 5.5-8.0 for initial pH (Table 6). The experimental design and the results are presented in Table 7.

Table 6. Experimental range and levels of the independent variables.

Independent variables	Range and levels				
	$-\alpha$	-1	0	1	$+\alpha$
Temperature	23.00	30.00	37.00	44.00	51.00
Inoculation volume	0.03	0.1	1.05	2.00	2.95
Incubation time	8	12	21	30	39
Initial pH	5.22	5.5	6.75	8.00	9.25

Table 7. Experimental design matrix using RSM with CCD and responses for cell counts.

Run No.	Variables				Response
	Temperature	Inoculation volume	Incubation time	Initial pH	Viable cell counts
	℃	%	hours	-	$\times 10^7$ CFU/ml
1	44	2	12	5.5	0.00016
2	37	0.03	21	6.75	262.333
3	30	2	30	5.5	128.670
4	37	1.05	21	6.75	286.667
5	30	0.1	12	8	81.1110
6	37	1.05	21	6.75	303.333
7	30	2	12	8	95.2222
8	23	1.05	21	6.75	93.3333
9	37	2.95	21	6.75	202.678
10	37	1.05	21	6.75	294.111
11	37	1.05	21	6.75	323.333
12	44	0.1	30	8	0.00017
13	30	2	12	5.5	151.556
14	37	1.05	21	9.25	19.4444
15	37	1.05	21	6.75	293.333
16	37	1.05	39	6.75	19.0000
17	44	0.1	12	8	0.00017
18	44	0.1	12	5.5	0.00011
19	44	2	30	8	0.00067
20	44	2	12	8	0.00063
21	30	0.1	30	5.5	48.2267
22	37	1.05	8	6.75	167.778
23	37	1.05	21	4.25	8.63300
24	37	1.05	21	6.75	297.889
25	30	0.1	30	8	86.7780
26	30	2	30	8	89.1110
27	44	2	30	5.5	0.00033
28	30	0.1	12	5.5	38.1111
29	44	0.1	30	5.5	0.00067
30	51	1.05	21	6.75	0.00017

According to the treatment combination described (Table 7), the viable cell counts with different culture conditions were optimized. By fitting the experimental responses with the least squares method, two second-order polynomial regression models were obtained as the linear model and the quadratic regression model for the response surface simplification.

The experimental results of the CCD were fitted with a second-order polynomial equation for estimation of biomass concentration:

$$Y = +302.99 - 37.7 \times A + 15.68 \times B - 12.95 \times C + 0.30 \times D - 13.15 \times AB + 0.83 \times AC + 0.90 \times AD - 2.80 \times BC - 11.09 \times BD + 0.77 \times CD - 69.59 \times A^2 - 41.81 \times B^2 - 57.91 \times C^2 - 77.74 \times D^2 \qquad (2)$$

Here Y is the predicted response of the bacterial viable cell counts ($\times 10^7$ cfu/ml), A, B, C and D are the actual values of temperature (℃)，inoculation volume (%), incubation time (hours) and initial pH, respectively.

Table 8. ANOVA analyse result.

	F-value	*p*-value	Sig
Model	54.67	<0.0001	***
A-temperature	66.47	<0.0001	***
B-inoculation volume	7.50	0.0152	**
C-incubation time	0.025	0.8767	NS
D-initial pH	4.298E-003	0.9486	NS

***P< 0.01, **P< 0.05, *P< 0.1.
$R^2 = 0.9510$, Pred-$R^2 = 0.9808$, Adj-$R^2 = 0.8675$.

Figure 18. Predicted and actual experimental value of optimal culture conditions predicted by RSM.
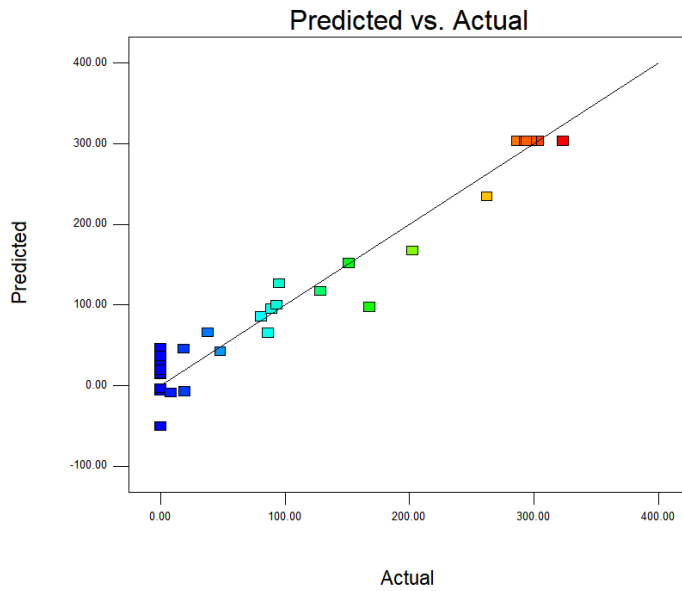
The results of ANOVA (Table 8) showed that "F-value" of the model was 54.67, and the "p-value" was < 0.0001, which indicates that the model was very significant. In this case, A-temperature and B-inoculation volume were significant model terms. However, the incubation time and the initial pH had no significant effect on the growth of the LP-GS1 in the range set by the experiment.

The interaction between the four independent variables was shown in Figure 19. It can be seen from the figure that four independent variables all showed the corresponding peak value, which is, the optimal condition.

The coefficient of determination (R^2) was calculated as 0.9510 for viable cell count which indicated good agreement between the experimental and the predicted values. The degree of discretization between the predicted value and the actual value was visualized by Figure 18. The pred-R^2 of 0.9808 was in reasonable agreement with adj-R^2 of 0.8675.
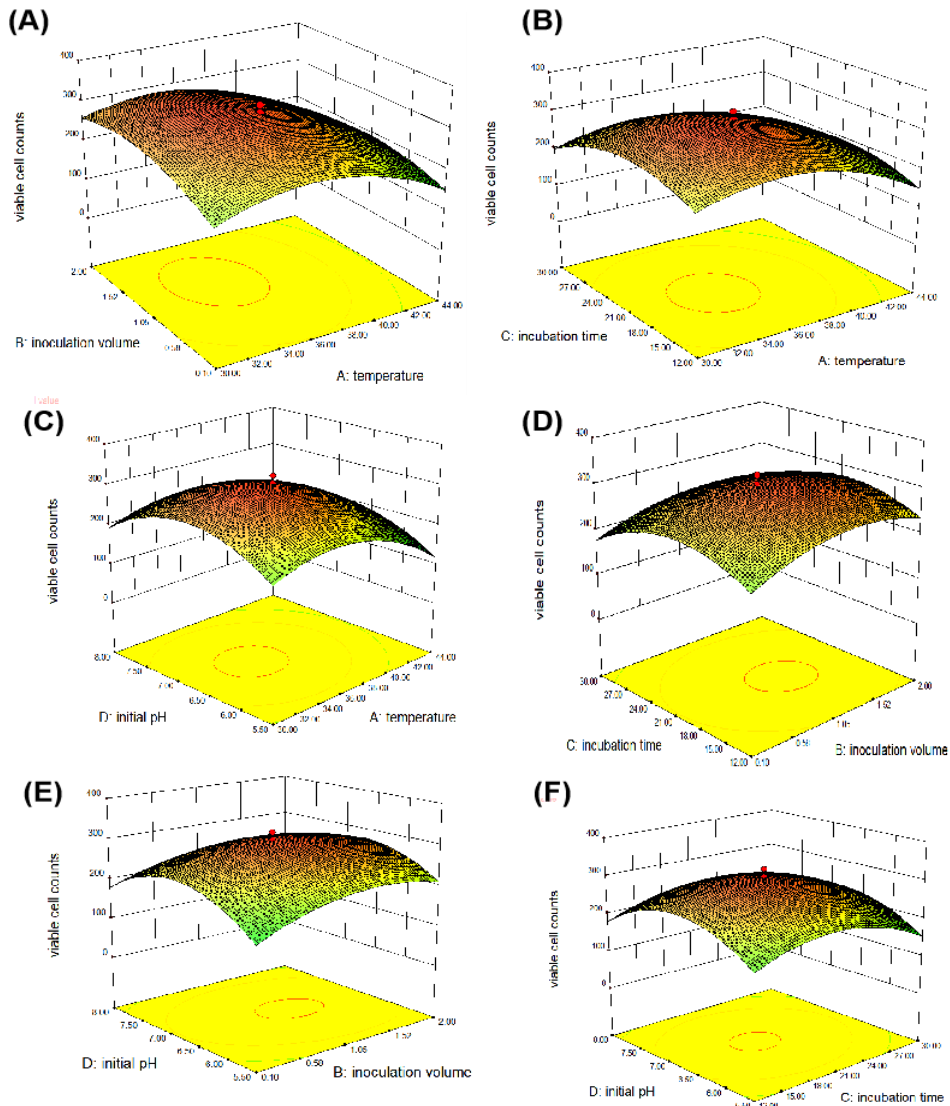
Figure 19. The effect of different candidates on the response. Interaction between (A) temperature and inoculation volume, (B) temperature and incubation time, (C) temperature and initial pH, (D) inoculation volume and incubation time, (E) inoculation volume and initial pH and (F) incubation time and initial pH.

Table 9. RSM prediction results .

Name	Goal	Lower limit	Upper limit
A-temperature	Is in range	30	44
B-inoculation volume	Is in range	0.1	2
C-incubation time	Is in range	12	30
D-initial pH	Is in range	5.5	8
Viable cell counts	Maximize	0.00011	323.333

		Solution			
Temperature(℃)	Inoculation volume(%)	Incubation time(hrs)	Initial pH	Viable cell counts ($\times 10^7$ cfu/ml)	Desirability
34.85	1.29	20.89	6.73	310.76	0.961

According to the results of statistical design, the optimum conditions were listed in Table 9 and the viable cell count was predicted to be 3.11×10^9 cfu/ml under this culture condition.

The theoretical values obtained by this method were tested in practice. The results showed that the actual number of bacteria reached 2.80×10^9 cfu/ml after being cultured in the optimized medium and culture conditions. Because there was no significant difference between the actual value and the theoretical value, the predicted results of RSM can be judged to be reliable. So far, the mass production conditions of LP-GS1 had been determined.

3) Optimization of freeze-drying process

In order to use probiotics as a final feed additive, it is required to go through a drying process. Due to the weak heat resistance of most lactic acid bacteria, including LP-GS1, freeze-drying method is mostly used in the process of product transformation.

(1) Selection of freezing methods before freeze drying

In order to ensure the survival rate to the greatest extent, three freezing methods before drying were compared: liquid nitrogen freezing method, -70℃ freezing method and continuous freezing method (freezing in the order of 4 ℃, -20 ℃ and -70 ℃ 20 minutes each).

It can be seen from the results of Figure 20 that independent of the type of cryoprotectants used, the survival rate in liquid nitrogen freezing treatment group is higher than that in -70 ℃ freezing treatment group, which is higher than that in continuous freezing method group. This may be due to the serious imbalance of intracellular and extracellular electrolytes during slow freezing, resulting in cell dehydration and death.
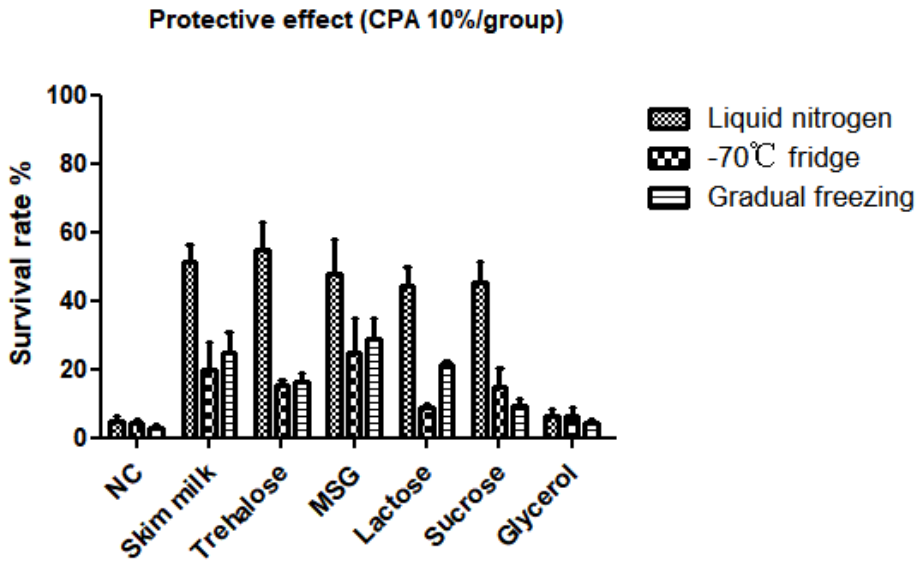
Figure 20. Protective efficiency of different cryoprotectant under different freezing conditions.

Liquid nitrogen: liquid nitrogen ultra-rapid freezing, -70℃ freezing method：rapid freezing at -70℃, continuous freezing method: slow freezing at 4 ℃,-20 ℃ and-70 ℃, respectively.

(2) Determination of cryoprotective additives by OFAT method

When frozen, the crystallization structure produced by the solidification of water inside and around the bacteria, these ice crystals will cause physical damage to the cell structure and lead to cell death. In order to alleviate this phenomenon, a variety of freeze-drying protectants are usually used to protect cells when freezing. In this experiment, the protective effect and product storage of many cryoprotectants widely used in the actual industry on LP-GS1 were observed.

The results showed that the protective effect of skim milk, trehalose and MSG was better than that of other CPAs (Figure 21 B,D), both at room temperature and in cold storage. In addition, the cell survival rate decreased rapidly when stored at room temperature (Figure 21 A,C). After 8 weeks, the survival rate of the room temperature group decreased to less than 10%, while the survival rate of most of the refrigerated treatment groups could be maintained at about 30%. Therefore, when storing the product, the ambient temperature will have an effect on the survival rate of bacteria, and according to the type of frozen drying protectant, the survival rate during storage was different.
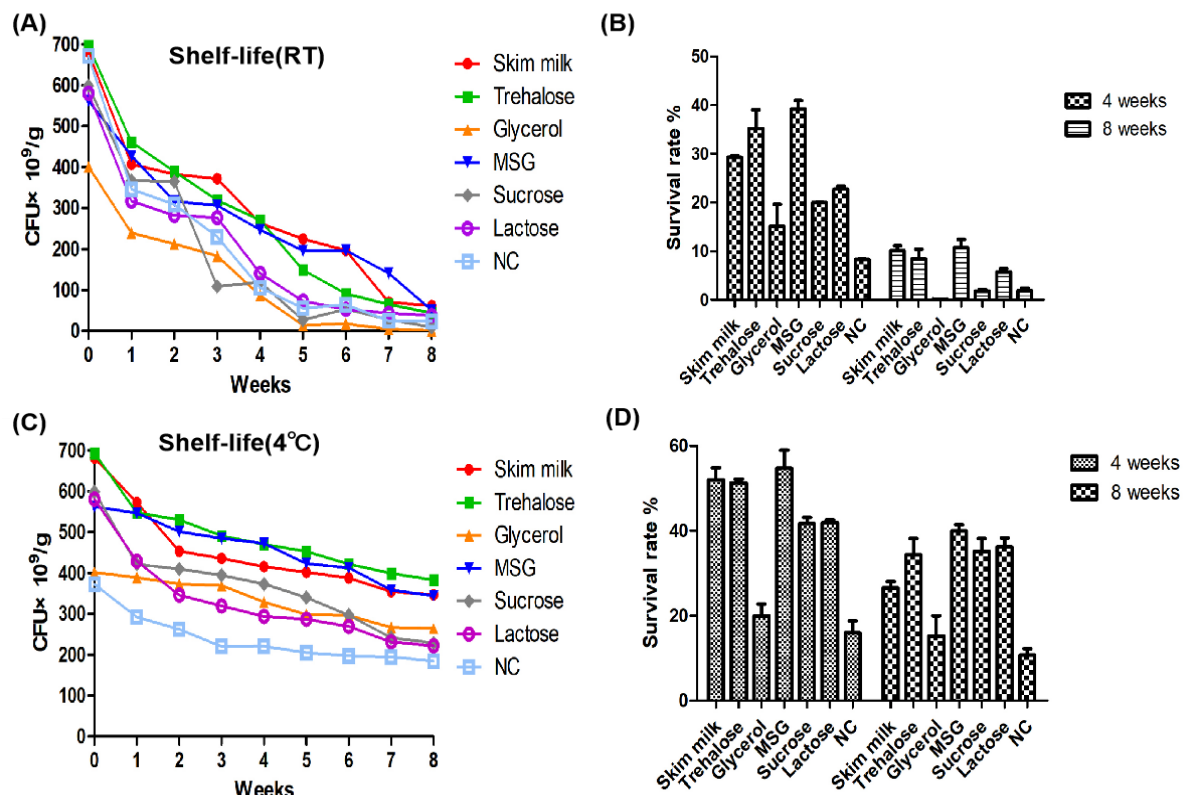
Figure 21. Effective Protection time of different cryoprotectant candidates. (A,C) Determination of the number of weekly

viable bacteria numbers treated with different cryoprotectant (B,D) 4-week and 8-week survival rate.

(3) Determination of cryoprotective additives by PBD method

In order to compare the protective effects of skim milk, trehalose, glycerol, MSG, sucrose and lactose in freeze-drying process, PBD design was adopted. Table 10 listed the variables used in the experimental design andthe response values of experimental design and cell survival rate are shown in Table 11.

The variable with confidence level above 95% is considered as significant parameter. Nine factors (Table 12) showed that X_1, X_2 and X_4 had a significant effect on cell survival rate, and this result was consistent with the phenomenon observed by OFAT, so variables X_1, X_2 and X_4 were determined to be the significant influencing factors. Variables X_3, X_5 and X_6 with confidence levels below 95% are considered unimportant and are not included in the next CCD experiment. The coefficient of determination (R^2) was calculated as 0.9519 which indicated good agreement between the experimental and the predicted values. The pred-R^2 of 0.7594 was in reasonable agreement with adj-R^2 of 0.9037.

Pareto diagram was sorted according to the frequency of events, which is a method to find out the main factors that affect the setting response value. As can be seen from Figure 22 that: the protective effect on cells for B, A, D, i.e., X_1, X_2 and X_4, was 1, 2, 3, respectively. At this point, skim milk, trehalose and MSG had been selected as the final optimization objects.

Table 10. Plackett-Burman experimental variable range.

Symbol	Variables	Experimental value		
		-1	**0**	**+1**
X_1	Skim milk	2	8.5	15
X_2	Trehalose	2	11	20
X_3	Glycerol	2	8.5	15
X_4	MSG	1	5.5	10
X_5	Sucrose	2	8.5	15
X_6	Lactose	2	8.5	15

Table 11. Experimental design and results of the Plackett-Burman test.

Run	X_1	X_2	X_3	X_4	X_5	X_6	Y_1 / survival rate %
1	+1	+1	-1	+1	+1	+1	91.6915
2	-1	+1	+1	-1	+1	+1	67.7532
3	+1	-1	+1	+1	+1	-1	75.4374
4	+1	+1	+1	-1	-1	-1	81.7847
5	-1	+1	-1	+1	+1	-1	63.2861
6	0	0	0	0	0	0	72.0158
7	-1	-1	-1	-1	-1	-1	38.4712
8	+1	-1	+1	+1	-1	+1	71.6776
9	+1	-1	-1	-1	+1	-1	53.5812
10	-1	+1	+1	+1	-1	-1	83.4816
11	+1	+1	-1	-1	-1	+1	80.7073
12	-1	-1	-1	+1	-1	+1	56.0156
13	-1	-1	+1	-1	+1	+1	41.6272

Table 12. Effects of the variables and statistical analysis of the

Plackett-Burman design.

Term	F value	*p*-value	Ranking	
X_1	34.63	0.0020	2	**
X_2	55.43	0.0007	1	***
X_3	4.60	0.0847	4	
X_4	19.22	0.0071	3	**
X_5	1.12	0.3380	5	
X_6	0.57	0.4826	6	
R^2: 0.9519		Adj-R^2: 0.9037		Pred-R^2: 0.7594

***P< 0.01, **P< 0.05, *P< 0.1.
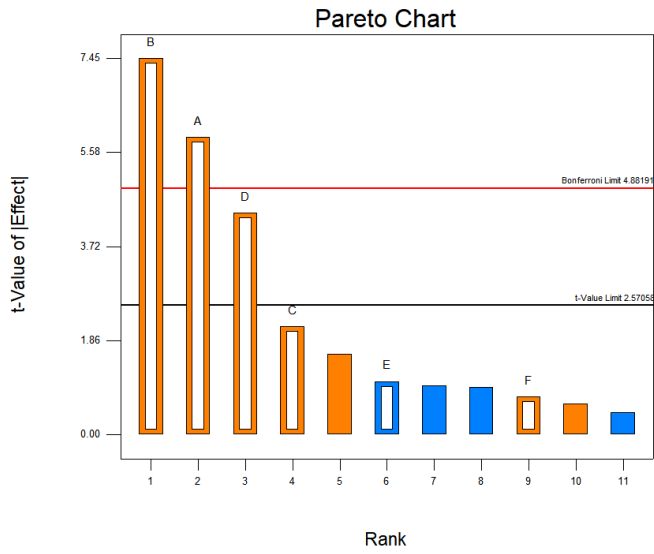
Figure 22. Pareto chart analysis results.

(4) Determination of the optimal ratio for cryoprotactants by CCD

Before the statistical analysis of CCD, in order to grasp the best concentration of the three cryoprotectants when used alone, the protective effect of skim milk, trehalose and MSG at different concentrations was preliminarily tested by OFAT method with the viable cell count and survival rate as the evaluation index, and the maximum concentration of cryoprotectants was determined when they were used alone.

The results from Figure 23 show that when the concentration of skim milk was 0 to 15%, the cell survival rate increased with the increase of dosage. While the addition concentration continues to increase, the survival rate presented a downward trend. This may be due to the high concentration of extracellular osmotic pressure leading to cell dehydration and death. Similarly, trehalose and MSG also had almost the highest survival after freeze-drying at concentrations of about 20% and 10%, respectively. Based on the results of this tesult, the research interval of the subsequent CCD experiment was set up (Table 13).
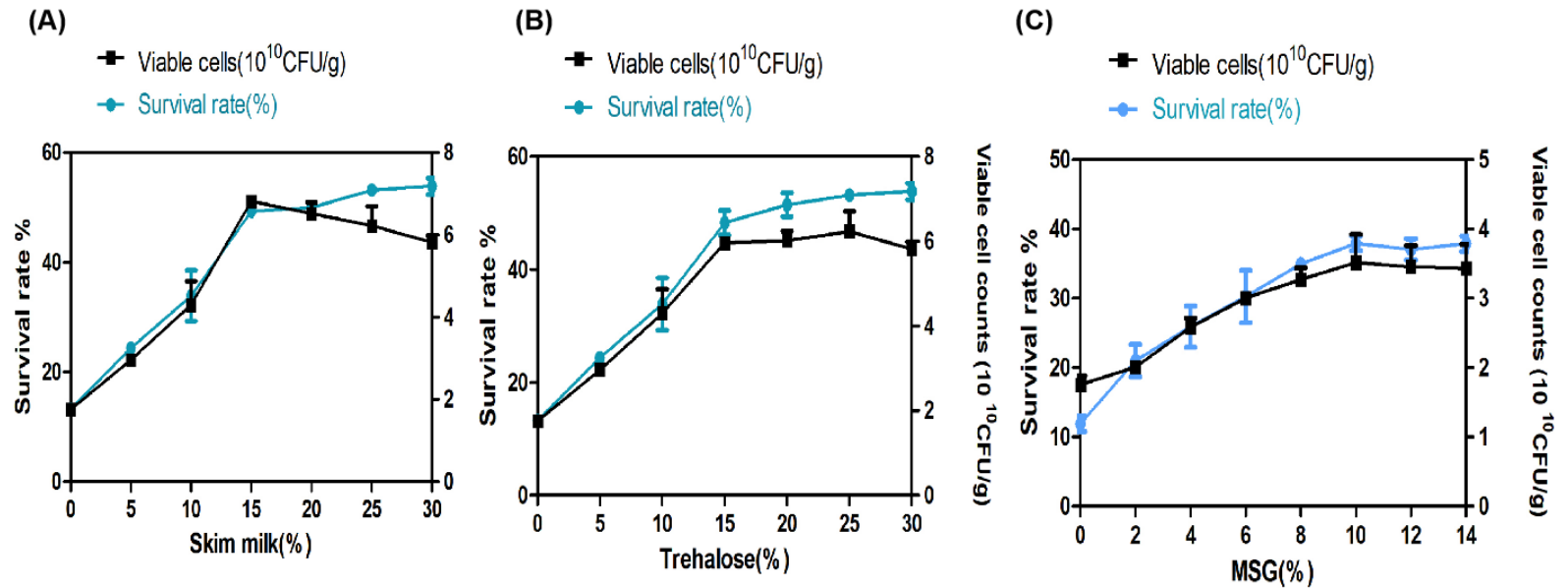
Figure 23. Grasp the optimal addition of cryoprotectant when it is used alone.

(A, B, C) Protective effects of skim milk, trehalose and MSG when it's mixed 1:1 (w/w) with LP-GS1, respectively.

Table 13. Experimental range and levels of the independent variables for CCD design.

Independent variables	Range and levels				
	$-\alpha$	-1	0	1	$+\alpha$
A-skim milk	0	2.00	8.50	15.00	19.43
B-trehalose	0	2.00	11.00	20.00	26.14
C-MSG	0	1.00	5.50	10.00	13.07

CCD analysis was carried out to predict the optimal proportion of the cryoprotectants which were determined by the OFAT and PBD experiments. Based on the cell survival rate, the ranges of the three variables were: skim milk 2-15%, trehalose 2-20%, MSG 1-10% (Table 13). The experimental design and results are shown in Table 14.

Table 14. Experimental design matrix using RSM with CCD and responses for cell survival rate and price.

Run No.	Variables			Responses	
	Skim milk (g/L)	Trehalose (g/L)	MSG (g/L)	Survival rate %	Price $/L
1	8.50	11.00	5.50	89.96	0.08784
2	8.50	11.00	5.50	92.45	0.08784
3	8.50	11.00	5.50	87.89	87.8900
4	8.50	26.14	5.50	76.24	0.13175
5	2.00	20.00	1.00	70.25	0.07048
6	15.00	2.00	10.00	74.59	0.10520
7	8.50	11.00	0.00	80.25	0.07508
8	2.00	20.00	10.00	79.36	0.09136
9	8.50	11.00	5.50	88.67	0.08784
10	8.50	11.00	5.50	87.58	0.08784
11	15.00	20.00	10.00	66.66	0.15740
12	2.00	2.00	10.00	45.25	0.03916
13	2.00	2.00	1.00	29.46	0.01828
14	8.50	0.00	5.50	64.97	0.05594
15	8.50	11.00	5.50	93.75	0.08784
16	15.00	20.00	1.00	76.88	0.13652
17	15.00	2.00	1.00	72.66	0.08432
18	8.50	11.00	13.07	75.56	0.10540
19	19.43	11.00	5.50	72.34	0.14336
20	0.00	11.00	5.50	65.66	0.04466

According to the treatment combination described in Table 14, the survival rate and price were optimized. By fitting the experimental responses with the least squares method, two second-order polynomial regression models were obtained as the linear model and the quadratic regression model for the response surface simplification.

The experimental results of the CCD were fitted with a second-order polynomial equation for estimation of biomass concentration:

$$Y = +90.30 + 7.50 \times A + 8.75 \times B + 1.74 \times C - 9.83 \times AB - 4.15 \times AC - 2.35 \times BC - 10.14 \times A^2 - 9.98 \times B^2 - 5.93 \times C^2 \tag{3}$$

Here Y is the predicted response of the bacterial survival rate after freeze-drying (%), A, B, and C are the actual values of skim milk (%), trehalose (%) and MSG (%), respectively.

Table 15. ANOVA analyse result.

Source	F-value	p-value	Sig
Model	94.28	<0.0001	significant
A-skim milk	123.97	<0.0001	***
B-trehalose	162.73	<0.0001	***
C-MSG	6.47	0.0291	***

***P< 0.01, **P< 0.05, *P< 0.1.
R^2 = 0.9884, Pred-R^2 = 0.9474, Adj-R^2 = 0.9779.

The *p*-value less than 0.001 indicated model terms were significant. In this case, A-skim milk, B-trehalose and C-MSG, all of the independent variables had significant effects on cell survival.
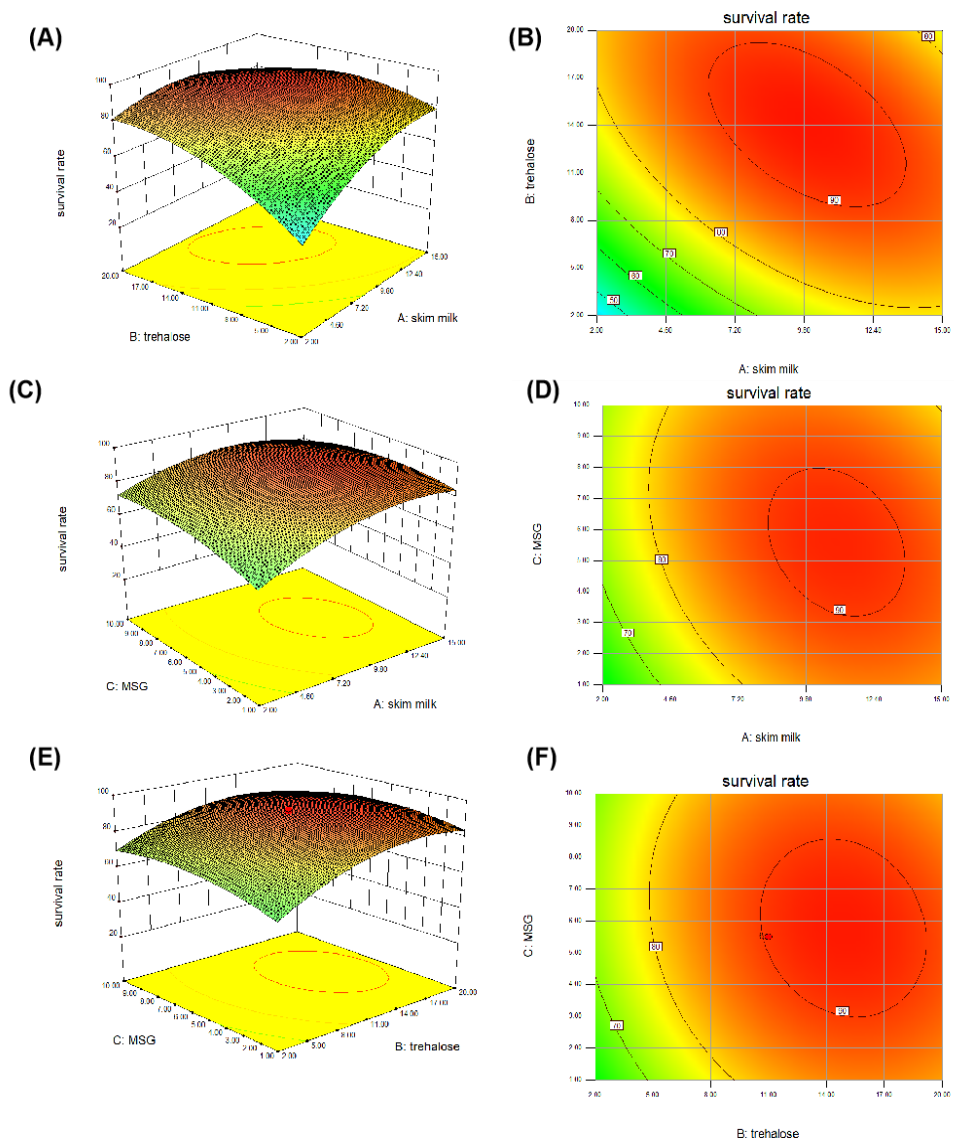
Figure 24. Three-dimensional response surface plots and two-dimensional contour plots for cell survival rate. Variable interactions of: (A) skim milk and trehalose, (B) skim ang MSG (C) trehalose and MSG.

Table 16. RSM prediction results.

Name	goal	Lower limit	Upper limit	
A: skim milk	Is in range	2	15	
B: trehalose	Is in range	2	20	
C: MSG	Is in range	1	10	
Survival rate	Maximize	29.46	93.75	
Price	Minimize	0.1828	1.574	
Solution				
Skim milk	Trehalose	MSG	Survival rate%	Price $/Kg
5.10	10.55	4.72	82.01	0.727

According to the results of statistical design, the optimum conditions were listed in Table 16 and the cell survival rate and price were predicted to be 82.10% and 0.727$/kg, respectively.

The theoretical values obtained by this method were tested in practice. The results showed that the actual cell survival rate was about 78.6% after freeze-drying of LP-GS1 with 5.1% skim milk, 10.55% trehalose and 4.72% MSG as cryoprotectants. Because there was no significant difference between the actual value and the theoretical value, the predicted results of RSM can be judged to be reliable.

4) Batch culture pH control

Because lactic acid bacteria can ferment carbohydrates to produce a large amount of lactic acid during growth, the medium pH is obviously decreased. In order to reduce the effect of pH on the growth of lactic acid bacteria, the gradient concentration of calcium carbonate was carried out under the optimal culture conditions. Based on the viable cell count, the optimum concentration of calcium carbonate was confirmed to be 1%.
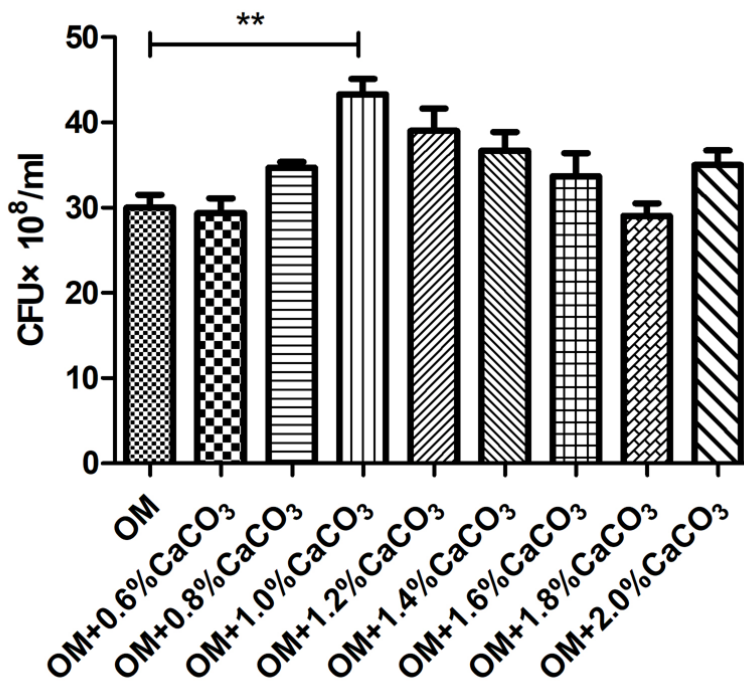
Figure 25. Medium pH regulation. Variation of cell numbers by the added level of CaCO3 in the optimum medium.

5) Comparison of existing production conditions and optimal production conditions

After optimizing the whole production process with RSM, the optimal production medium and culture conditions were determined. In order to compare the difference between the number of bacteria cultured under the optimal condition (OC) and the laboratory standard condition (LS), a comparative experiment was carried out.

According to the results, after adding 1% calcium carbonate, the yield of general laboratory culture conditions was about 3.58×10^9 cfu/ml, while the yield of optimized culture conditions was about 4.75×10^9 cfu/ml. Therefore, the optimal production conditions could not only save the production cost, but also promote the growth of LP-GS1.
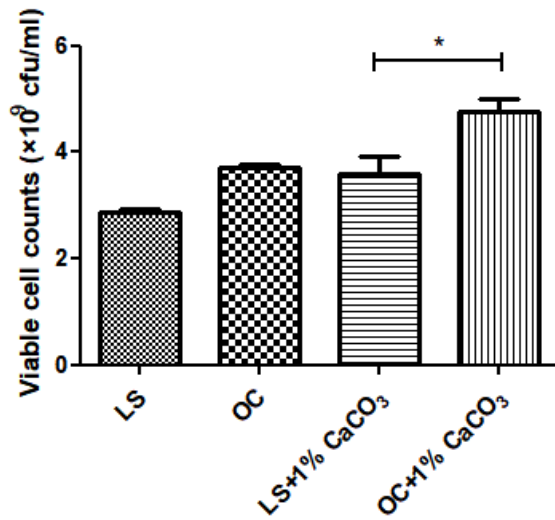
Laboratory standard (LS): 37℃, initial pH 6.5, 0.1% inoculation, 12hrs culture

Optimal conditions (OC): 34.85℃, initial pH 6.73, 1.29% inoculation, 20.89hrs culture

Figure 26. Final growth performance comparison. Comparison of bacterial production by using general laboratory incubation conditions (LS) and optimized incubation conditions (OC).

6) Changes of antibacterial ability before and after freeze-drying

After freeze-drying, the original antibacterial ability of lactic acid bacteria will be changed due to the damage to a certain extent and the inhibition of enzyme activity at low temperature. In order to verify that LP-GS1 still has stronger antibacterial ability against *E.coli* k99 than wild type after freeze-drying, the following experiments were conducted.

As a result, the antibacterial ability decreased slightly after freezing and drying, but it was a simultaneous phenomenon between the two bacteria and there was no significant difference. Therefore, it can be judged that the antibacterial ability of LP-GS1 was still about 2.26 times higher than that of WT after freeze-drying.
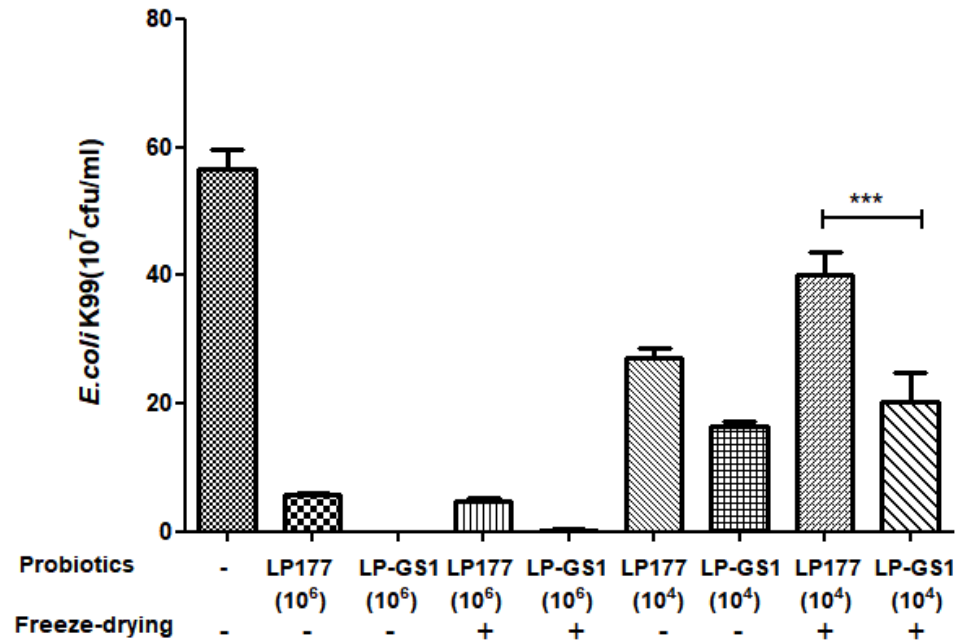
Figure 27. Changes of antibacterial ability before and after freeze-drying

7) Investigation of the shelf-life

Through previous experiments, the combination of growth medium, incubation conditions, and freezing dry protection agents was optimized to secure the necessary experimental conditions for production with actual feed additive. The storage of probiotics produced using the conditions was checked for eight weeks based on the survival rate of 0 weeks. Through this process, the survival rate of 4°C refrigerated storage was confirmed at 70.2% and 49.2% for room temperature storage in the fourth week which is considered to be the general distribution period of feed additives. In addition, it was confirmed that the survival rate for 4°C refrigerated storage was 45.0% and 14.6% for room temperature storage was recorded after 8 weeks, which was twice as much as stored at room temperature.
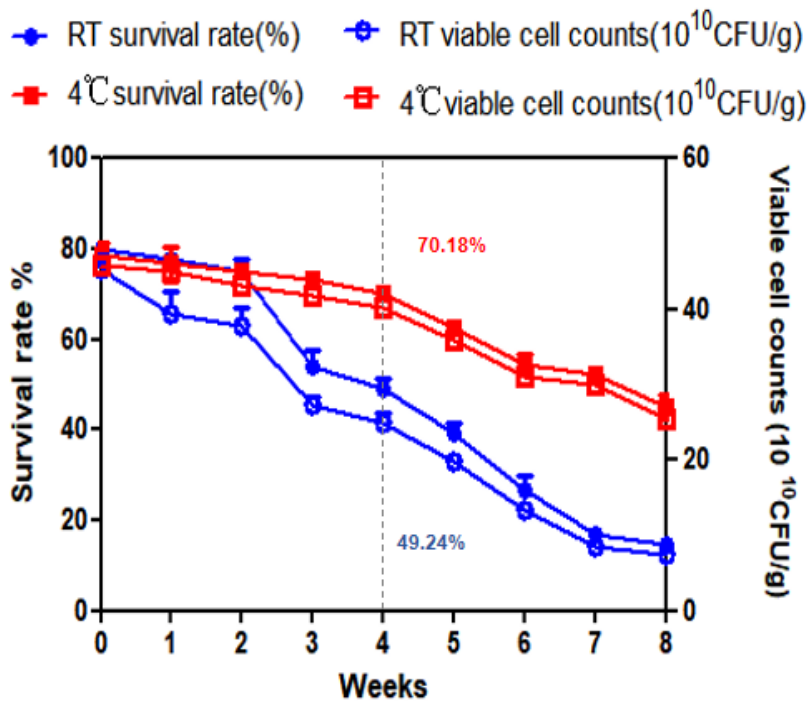
Figure 28. Shelf-life of LP-GS1 after freeze-drying when stored at RT and 4℃. Variation of bacterial survival rate and viable counts for 8 weeks in combination of optimized growth medium composition, incubation conditions and cryoprotectants.

4. Discussion

Lactic acid bacteria (LAB) are one of the most industrially important groups of bacteria, and they comprise a heterogeneous group of gram-positive catalase-negative, nonsporulating rods or cocci, producing lactate as amajor end-product of carbohydrate metabolism (Pfeiler and Klaenhammer 2007). However, the nutrient requirements of lactobacilli are complex and either strain or species-dependent in the fermentation of carbohydrates, nitrogen sources, fatty acids as well asgrowth factors (Hammes and Hertel 2006).

According to the results of previous studies, lactic acid bacteria, including *L. plantarum*, cannot produce some necessary growth molecules, such as purine, pyrimidine and ribose 5-phosphate (Hammes and Hertel 2006). Therefore, it can be said that *L. plantarum* is more dependent on organic nitrogen sources than on inorganic nitrogen sources (such as yeast extracts and pancreatic peptone). Yeast extract is a nitrogen source rich in nitrogen, vitamin B, purine and pyrimidine, and it has been widely used in the culture of lactic acid bacteria (Aeschlimann and von Stockar 1990). Its effect on the growth of lactic acid bacteria has been proven many times. Amrane and Prigent reported that the yeast extract concentration (ranging between 20 and 30 g/l) enhanced biomass production and growth rate during homolactic fermentation of L. helveticus (Amrane and Prigent 1998).

Because of the poor heat resistance of lactic acid bacteria, freeze-drying technology is widely used in their long-term storage. Microbes are exposed to a variety of pressures, such as freezing, dehydration and high pressure. These stressful environments lead to a reduction in viability, stability, and metabolic activity (Carvalho et al. 2004).

For general *Lactobacillus* production, the bacterial inoculum at a ratio of 0.1% (v/v) at 37 °C using commercial medium MRS is incubated for more than 12 hours, and 10% skim milk is used as a freeze drying cryoprotectant for bacteria. In this study, important factors were selected through OFAT and PBD for each component by dividing the incubation conditions into a combination of the growth medium, production conditions and freeze-drying cryoprotectants to optimize the production of LP-GS1. In the end, the optimization process was carried out with RSM analysis using CCD.

First, it was verified that nutrients dominated by carbon and nitrogen sources were the main factors affecting the growth of the bacteria. Based on this, the cost of creating optimized badges was reduced to 0.0779 $/L, which is 26.14% lower than the existing MRS prices (0.298 $/L). It was also confirmed that MRS costs $0.09 when it produces the same number of cells 1×10^{12} CFU, while optimized medium can be produced more economically at $0.02 or at about 22.22% of it.

Next, it was confirmed that the incubation temperature and the inoculation rate were the main contributors in the incubation conditions and that it was

possible to produce LP-GS1 cells at a maximum level of 3.7×10^9 CFU/ml. This was an increase of about 29% compared to the production (2.88×10^9 CFU/ml) under existing laboratory incubation conditions (37 ℃; initial pH 6.5; inoculation ratio 0.1%; 12-hour incubation).

Finally, in order to reduce the damage of ice crystal formation to cells during freeze-drying, the optimization of cryoprotectants were realized by PBD and CCD analysis. Compared with the protective effect of the existing laboratory cryoprotectant (1% skim milk), the survival rate of the cell after freeze-drying is increased by 2.67 times when the optimal combination (5.1% skim milk, 10.55 trehalose and 4.72% MSG) was used.

In addition, to prevent the inhibitory effect on bacterial growth due to the production of lactic acid, the optimal concentration was found to be 1% by adding calcium carbonate (CaCO3) by level to the Optimum media (OM) optimized by the previous experiment (Figure 25). After checking the bacterial production, it was found that production increased to about 3.58×10^9 CFU/ml for the general laboratory incubation conditions and 4.75×10^9 CFU/ml for the optimized incubation conditions.

It took 0.2304\$ to produce 1×10^{12} CFU of bacteria for the production of common lactobacillus, while this study confirmed that the optimal mix of the growth media, incubation conditions, and freeze - drying protectants cost \$0.355 to economically produce LP-GS1 at about 15.41% of the previous level.

This study was confirmed the possibility of the industrial mass production of LP-GS1.

Table 17. Summary of study 1.

Contents		Results
(Study 1-1) Mass production	Optimization of culture medium	- Optimum medium(C&N): 6.72%molasses, 1.21% yeast extract
		- Cost of 1L: optimum medium 0.0779$, MRS 0.298$ (73.86% saved↓)
	Optimization of culture conditions	- Optimum culture condition: temperature 34.85℃, inoculation volume 1.29 %, incubation time 20.89 hrs, pH 6.73, $CaCO_3$: 1.0%
		-Cost of 1×10^{12} CFU production: MRS 0.09$, OM 0.02$ (77.73%saved↓)
(Study1-2) Survival rate enhancement	Optimization of freeze-drying conditions	- Skim milk 5.11%, trehalose 10.56%, MSG 4.71%
		- Survival rate compaired with conventional method: 80%, 2.67 times higher than traditional method↑
		- Shelf-life: 70% at 4℃ after 4 weeks, 49% at RT after 4 weeks
Antimicrobial capacity against *E.coli* K99		- Antimicrobial capacity: 1.98 times higher↑ than wild type
		- The antibacterial ability was maintained after freeze-drying

Study 2. *In vivo* efficacy and antibacterial ability as an alternative probiotic substitute for feed additives of *Lactobacillus plantarum* GS1

1. Introduction

Weanling is very stressful for young animals, especially when it is accompanied by changes in society, body and nutrition (O'Loughlin et al. 2011). At weanling, the feed of piglets changes, which may break the microbial balance in gastrointestinal tract and enable a variety of pathogens represented by E. coli to settle in the intestine (Jensen 1998). ETEC strains were first recognized as causes of diarrheal disease in piglets, where the disease continues to cause lethal infection in newborn animals (NATARO and KAPER 1994). An impaired gut microbiota composition during the neonatal period may lead to chaotic growth of bacterial species after weaning (Mulder et al. 2009).

Growth performance is often used to indicate changes in body weight over time so that intake and diet composition can match it and to assess the genetic potential of animals (Lopez et al., 2000). Growth is influenced by genotype, environment, feeds and many other factors in pigs (Denis Bastianelli 1997). Environmental stress includes separation from mothers, adaptation to a new environment, changes in food, etc. Under this environment, young pigs will be

highly stressed because of a lot of pressure, resulting in a large number of stress reactions, which will eventually lead to disorder of the intestinal microbiota, the production of inflammation and ultimately to a decline in immunity and a decrease in growth performance (McGlone and Curtis 1985) and an increase in cortisol concentration (R. Dantzer 1980). Therefore, environmental stress will eventually have a negative effect on the growth performance of young pigs.

The aim of this study was to evaluate the effect of LP-GS1 as a feed additive on growth after weaning in piglets to verify its effect as an antibiotic substitute for probiotics.
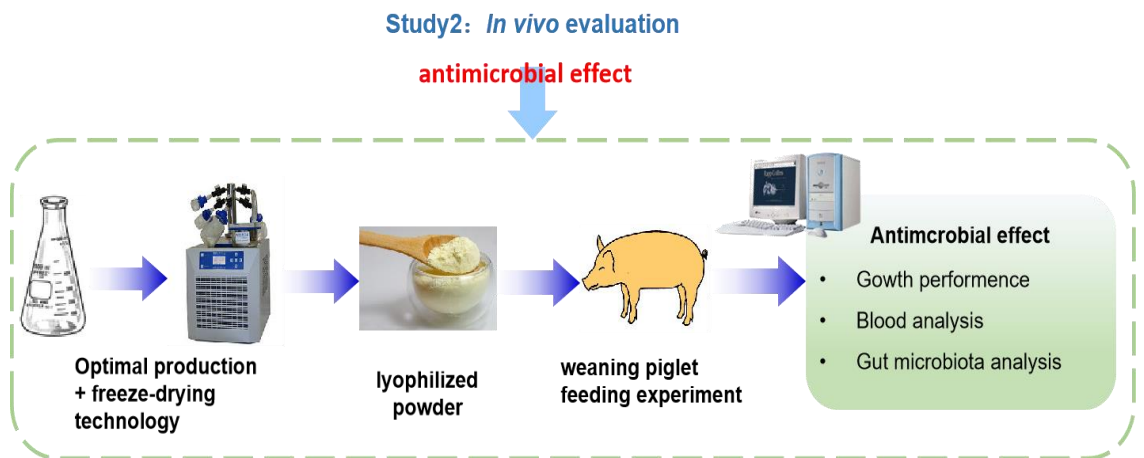
Figure 29.Graphical abstract of study 2.

2. Materials and Methods

1) Preparation of probiotic feed additives

(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 seed culture, Rogosa and Sharpe *agar* (MRS) was purchased from BD Difco (Sparks, MD, USA). For bacterial mass production, the optimal medium which was selected in study 1 was used. And pH of medium was measured using ORION STAR A211 pH-meter (Thermo Scientific, USA).

(2) Microbial cultivation

In seed culture, streaking was performed on MRS agar plate using the bacteria stock which is stored in -70℃ deep-freezer. After culture at 37℃ for 24 hours, one colony was selected and inoculated in 10ml MRS broth and cultured with overnight.

For large scale batch culture, both of LP 177 and LP-GS1 strains were cultured in LP-GS1 optimal medium which is determined in study 1. Bacteria were cultured at 34.85 °C in the incubator for 24 h. The initial pH was 6.73, the inoculum size was 1.29%, the culture time was 20.89 h, and the addition amount of calcium carbonate was 1%.

(3) Bacterial freeze-drying

The bacteria culture solution was centrifuged at 4℃, 6000 rpm for 15 minutes and the supernatant was discarded to obtain the cultured cells. The cells were then suspended in 1X PBS solution at the ratio of 1:10 (w/w) and washing under the same centrifugal conditions for 3 times. Finally, cells were mixed with cryoprotectants (skim milk 5.11%, trehalose 10.56% and MSG 4.71%) in the proportion of 1:1 (w/w). Then, after rapid pre-freezing in liquid nitrogen, samples were desiccated in a vacuum freeze-dryer at a condenser temperature of − 80°C.

2) Animals and experimental design

A total of 120 28-day-weaned piglets (Landrace–Yorkshire–Duroc) were randomly allocated to four treatments with three replicates of 10 piglets in each treatment and raised for 5 weeks in cages (Figure 30 and Table 18). The Institutional Animal Care and Use Committee at Seoul National University approved the animal experiments (SNU-180904-2-1). The piglets were housed with ad libitum access to feed and water. Basal diets changed in different growth stage and the compositions were analysed (Table19, Table 20 and Table 21).

All piglets received the basal diet as powder feed. NC group was only administered with the commercial feed. PC group was administered with 100mg colistin per kilogram feed. The T1 and T2 group was administered a single dose of 2×10^9 CFU / kg of daily feed with *L. plantarum* 177 and *L. plantarum* GS1

which was freeze dried and mixed with calcium carbonate and then mix with the basal diet, respectively.

Diets were fed during the whole experimental period. Body weight (BW), feed intake (FI), fecal and blood samples were measured on days 0, 14, 28 and 35, respectively. Then BW and FI were used to calculate the ADG, ADFI and FCR.
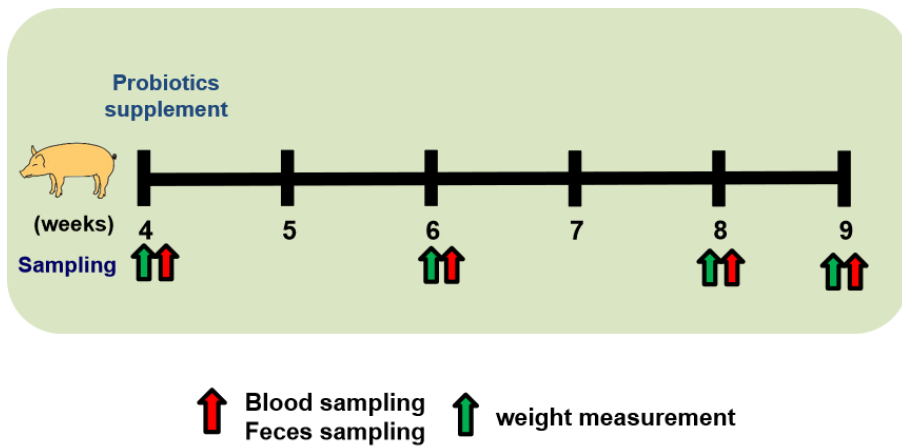
Figure 30. *In vivo* **experimental schedule.**

28-day-weaned piglets were used for the feeding experiment during 5 weeks. The blood and feces were sampled in 0, 2nd, 4th, 5th weeks (red arrow). The weight of the feed and body weight was also measured (green arrow).

Table 18. Experimental groups and design.

Group	Treatment	Additive amount(/feed kg)	Pen	Piglets/ pen	Piglets	Experimental date (day)
NC	Untreated	-	3	10	30	35
PC	Antibiotic	100mg	3	10	30	35
T1	LP-177	2×10^9 CFU	3	10	30	35
T2	LP-GS1	2×10^9 CFU	3	10	30	35
Total	-	-	12	40	120	-

Table 19. Composition of the basal diet (1 to 14 days).

Item	Group			
	NC	PC	T1	T2
Moisture (%)	11.25	11.34	11.17	11.70
Crude protein (%)	16.95	17.84	17.69	17.58
Crude fat (%)	5.73	5.64	5.80	5.77
Crude fiber (%)	2.48	2.56	2.20	2.40
Crude ash (%)	5.19	4.94	5.08	5.06
Ca (%)	0.74	0.70	0.73	0.74
P (%)	0.53	0.53	0.52	0.53
Digestable energy	3.7 Mcal/kg			

Table 20. Composition of the basal diet (15 to 28 days).

Item	Group			
	NC	PC	T1	T2
Moisture (%)	12.41	12.56	12.45	12.36
Crude protein (%)	19.38	19.38	17.75	18.33
Crude fat (%)	5.29	5.21	5.03	5.02
Crude fiber (%)	2.52	2.90	2.48	2.34
Crude ash (%)	6.69	6.80	7.10	7.19
Ca (%)	0.84	0.84	0.91	0.99
P (%)	0.70	0.67	0.70	0.69
Digestable energy	3.55 Mcal/kg			

Table 21. Composition of the basal diet (29 to 35 days).

Item	Group			
	NC	PC	T1	T2
Moisture (%)	15.37	15.14	15.24	15.34
Crude protein (%)	16.44	17.98	17.34	15.68
Crude fat (%)	4.97	4.90	4.69	4.09
Crude fiber (%)	2.36	2.20	2.38	2.39
Crude ash (%)	8.69	6.45	6.48	5.43
Ca (%)	1.60	0.75	1.03	0.81
P (%)	0.46	0.32	0.42	0.37
Digestable energy	3.50 Mcal/kg			

3) DNA extraction and sequencing

DNA was extracted according to the manufacturer's protocol from 2000 mg of each fecal sample using the AccuPrep® Stool DNA extraction kit (Bioneer, Daejeon, Republic of Korea) and was stored by storage at - 20 °C until further analysis.

4) Quantitative real-time PCR

For the quantitative real-time PCR, primers were used to detect the diversification of *Lactobacillus spp., Lactobacillus Plantarum, Bifidobacterium spp., L. acidophilus, L. casei, E. coli* and ETEC. The primer sequences are listed in Table 22.

Quantitative real-time PCR (qRT-PCR) was performed with SYBR qPCR Mix using one-step real-time PCR. The amplification reactions were carried out in a total volume of 20 μl containing TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Daejeon, Korea), both primers and 5 ng of bacterial gDNA. Amplification (2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 5 s at 95 °C, 60 s at 60 °C) and detection were carried out on Bio-Rad's CFX96 Touch real-time PCR Detection System (Bio-Rad, CA, USA). To determine the specificity of the primers, a melting analysis of the DNA fragments was performed according to the manufacturer's instructions.

For relative quantification, 1μg of 16S rRNA cDNA was used as the reference gene when measuring 1μg target gene. According to the Ct value, the relative expression of the target gene was calculated by ΔΔCt method. The

target gene expression was standardized as the relative expression of 16s rRNA as an internal control in each sample and the data were presented as the relative expression.

Table 22. Primers for the identification of bacterial species by qPCR.

Target strains/genes	Sequences (5'-3')	Primer sizes (mer)	Tm (℃)	%GC	Product sizes	References
E.coli	F: GGACTTGATAACGTACCCGC	20	59.5	55	449	Mora et al., 1997
	R: GTTCCGTCTTGCATTTGACC	20	57.4	50		
ETEC	F: AATGCATCTTATGCCGGTG	19	57.2	47	160	Byun et al., 2012
	R: TCTTTGAATCTGTCCGAGAATATC	24	56.1	37		
Lactobacillus spp.	F: AGGTTTTGCTAATCAAGGGGG	21	57.3	47	110	Byun et al., 2013
	R: GTGCTATTCGACGCCTTAACC	21	59.8	52		
Lactobacillus Plantarum	F: GTAAGTTACACTATAAAAGCACCGTCG	27	59.8	40	106	Sharma, 2003
	R: TCTGTGTGGATGGTAATAAATTTTTG	26	55	30		
Bifidobacterium spp.	F: CGCGTCYGGTGTGAAAG	16	57.1	62	244	Delroisse J.-M. et al. 2008
	R: CCCCACATCCAGCATCCA	18	61.7	61		
L. acidophilus	F: TGGATGCCTTGGCACTAGGA	20	62.7	55	92	Haarman and Knol, 2006
	R: AAATCTCCGGATCAAAGCTTACTTAT	26	57.5	34		
L. casei	F: CTATAAGTAAGCTTTGATCCGGAGATTT	28	61.7	36	173	Haarman and Knol, 2006
	R: CTTCCTGCGGGACTGAGATGT	21	56.5	57		
16s rRNA	F: CCTACGGGAGGCAGCAG	17	61.9	70	450	Takahashi et al. 2014
	R: GGACTACHVGGGTWTCTAAT	21	50.4	47		

5) Blood component analysis

Blood metabolites were analyzed using a BS-400 chemistry analyzer (Mindray, Shenzhen, China) according to the manufacturer's protocol. For protein metabolism the total protein (TP, g/dl), blood urea nitrogen (BUN, mg/dl), albumin (Alb, g/dl) and globulin (Glb, g/dl); for mineral metabolism, calcium (Ca, mg/dl) and inorganic phosphorus (iP, mg/dl); For energy metabolism, glucose (Glu, mg/dl), total cholesterol (T-Chol, mg/dl), non-esterified fatty acid (NEFA, mmol/l), and triglyceride (TG, mg/dl); for liver function, aspartate aminotransferase (AST, U/l) and gamma-glutamyltransferase (GGT, U/l); for kidney function, creatinine (Crea, mg/dl). Characteristics of blood metabolites in piglet experiment were indicated in Table 25.

6) 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) Growth performance and diarrheal incidence

In many studies, *Lactobacillus plantarum* has been reported to be able to significantly improve various growth indicators in weaning piglets which include ADG, ADFI and FCR, diarrhea and intestinal environment and so on.

In order to evaluate the effect of *Lactobacillus plantarum* as a probiotic and compare the difference of biological efficiency between wild type (LP 177) and genome shuffling mutant (LP-GS1), we carried out *in vivo* experiment. The experiment included four treatment groups: negative control (NC, no beneficial bacteria), positive control (PC, colistin treatment), LP 177 (T1, wild type treatment) and LP-GS1 (T2, wild typ genome shuffling mutant treatment). The feeding experiment was carried out for five weeks after weanling of four-week-old piglets.

The results of growth performance parameters (Table 23) showed that the BW in T2 treatment group was the highest among the four treatment groups from the second week of the experiment, but there was no significant difference. Additionally, compared with NC, the FCR of T1 and T2 treatment

group all decreased. This suggests that *Lactobacillus plantarum*, as a probiotics, is beneficial to promote the growth of weaning piglets, and LP-GS1 has a better effect on promoting growth than wild type.

In order to study the ability of *Lactobacillus plantarum* to improve diarrhea in weanling piglets, we examined diarrhea and mortality rate in each treatment group every day. The results showed that the incidence of diarrhea in the four groups all increased with the development of the experiment. However, from the 3rd week, the incidence of diarrhea in PC and T2 treatment group was significantly lower than that in NC treatment group. T1 was also improved compared with NC, but there was no significant difference. In addition, there was no death happened in antibiotic treatment group, but T1 and T2 decreased the mortality rate of weaning piglets compared with NC.

During the whole experiment, the treatment group with probiotics reduced the incidence of diarrhea and improved the growth performance, suggesting that *L. Plantarum* is a potential probiotics for pigs. In addition, T2 had a better effect on reducing the incidence of diarrhea than T1, indicating that genome shuffling mutant has a stronger ability to maintain intestinal health after weanling than wild type.

Table 23. Effects of probiotics on growth performance and feed intake of weaning piglets.

Parameters		Treatment				P value
		NC	PC	T1	T2	
BW (kg)	0 d	9.55±1.94	9.55±1.90	9.56±1.96	9.56±1.92	0.9986
	14 d	13.77±3.58	13.80±3.29	13.53±3.42	14.11±2.99	0.9341
	28 d	20.88±4.16	20.55±4.22	19.88±4.60	21.08±4.25	0.7080
	35 d	22.88±4.66	23.03±3.77	21.99±5.02	23.22±3.83	0.7250
0 to 14 d	ADG, kg	0.30±0.17	0.30±0.13	0.29±0.14	0.32±0.11	0.8082
	ADFI, kg	0.43±0.03	0.42±0.04	0.41±0.02	0.42±0.02	0.9142
	FCR	1.41±0.11	1.40±0.13	1.40±0.08	1.30±0.06	0.6034
14 to 28 d	ADG, kg	0.54±0.22	0.55±0.21	0.49±0.13	0.54±0.22	0.6185
	ADFI, kg	0.76±0.09	0.75±0.10	0.70±0.11	0.74±0.09	0.9334
	FCR	1.39±0.17	1.36±0.19	1.43±0.23	1.36±0.16	0.9808
28 to 35d	ADG, kg	0.33±0.33	0.37±0.48	0.35±0.25	0.36±0.43	0.9965
	ADFI, kg	1.04±0.08	1.09±0.07	1.03±0.07	1.09±0.06	0.7173
	FCR	3.13±0.24	2.95±0.21	2.97±0.20	3.07±0.16	0.7766

Data are presented as the means±SD. One-way ANOVA with Tukey's post hoc test was used (*$p < 0.05$).

BW, Body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Table 24. Diarrhea score and mortality rate.

Score		Treatment				*P* value
		NC	PC	T1	T2	
Diarrhea	0 to 7 d	0.29±0.26	0.13±0.16	0.58±0.49	0.29±0.26	0.0788
	7 to 14 d	0.86±0.50	0.67±0.13	1.10±0.49	0.57±0.29	0.1715
	14 to 21 d	1.08±0.55	0.46±0.29	1.04±0.31	0.75±0.32	0.0160**
	21 to 28 d	1.52±0.35	0.62±0.28	1.19±0.16	0.85±0.28	<0.0001***
	28 to 35 d	1.48±0.24	0.71±0.21	1.33±0.40	0.95±0.21	0.0003***
	Mean	1.01±0.61	0.49±0.34	1.01±0.45	0.68±0.36	
Mortality rate	0-35d	6.67%	0	3.33%	3.33%	

Data are presented as the means±SD. One-way ANOVA with Tukey's post hoc test was used (*p<0.05).

FCS (fecal consistency score): 0-normal feces, 1-soft feces, 2-mild diarrhea, 3-severe diarrhea. This indicator was checked every day.
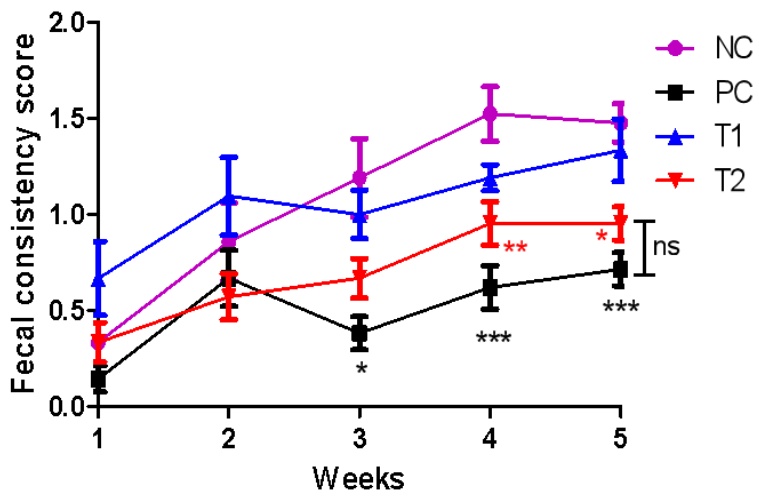
Figure 31. Diarrhea index in weaning piglets.

2) Microbial analysis in weaning piglets

In intestinal microbial analysis, viable cell count and real-time PCR (qPCR) were used to identify the number of specific microorganisms in fecal samples.

In order to track the changes of microorganisms in the intestine of piglets after weanling, six individuals in each group were randomly selected for fecal sampling at 0, 2, 4 and 5 weeks. MacConkey agar, MRS agar and Nutrient agar were used to count the viable cells in the feces collected.

On the first day of the experiment, the content of G$^-$ bacteria in feces of all treatment groups was basically similar, about 6.06 log^{10} cfu/g. With the change of time, the G$^-$ bacteria content in the feces of NC control group increased gradually, and the other three groups showed a tendency to decrease gradually (Figure 32). Among them, the downward trend of PC antibiotic treatment group was the most obvious, followed by T2 treatment group, while T1 only slightly decreased. This showed that LP-GS1 had better antibacterial effect than LP 177 when used as feed additive.
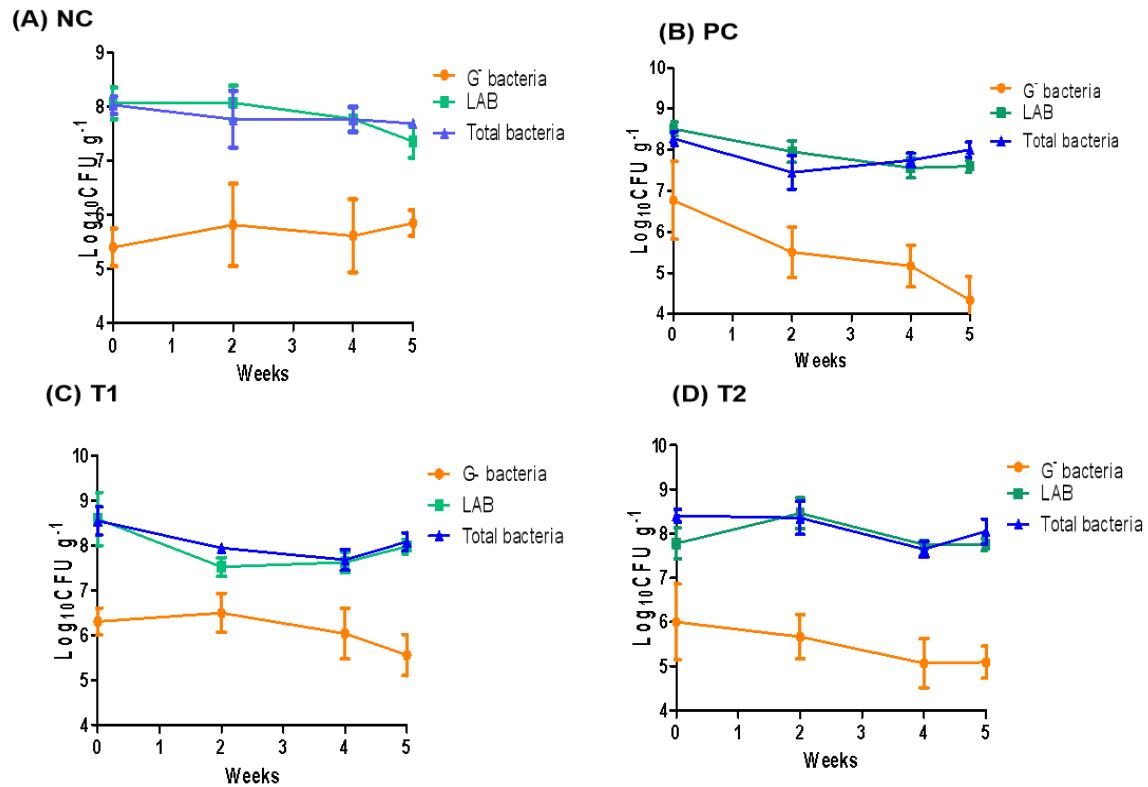
Figure 32. Number of G⁻ bacteria, LAB and total bacteria cells in feces.

(A-D) Microbial changes of gram negative bacteria, lactic acid bacteria and total bacteria in feces during 5 weeks in NC, PC, T1 and T2 gruops use MacConkey agar, MRS agar and Nutrient agar.

130
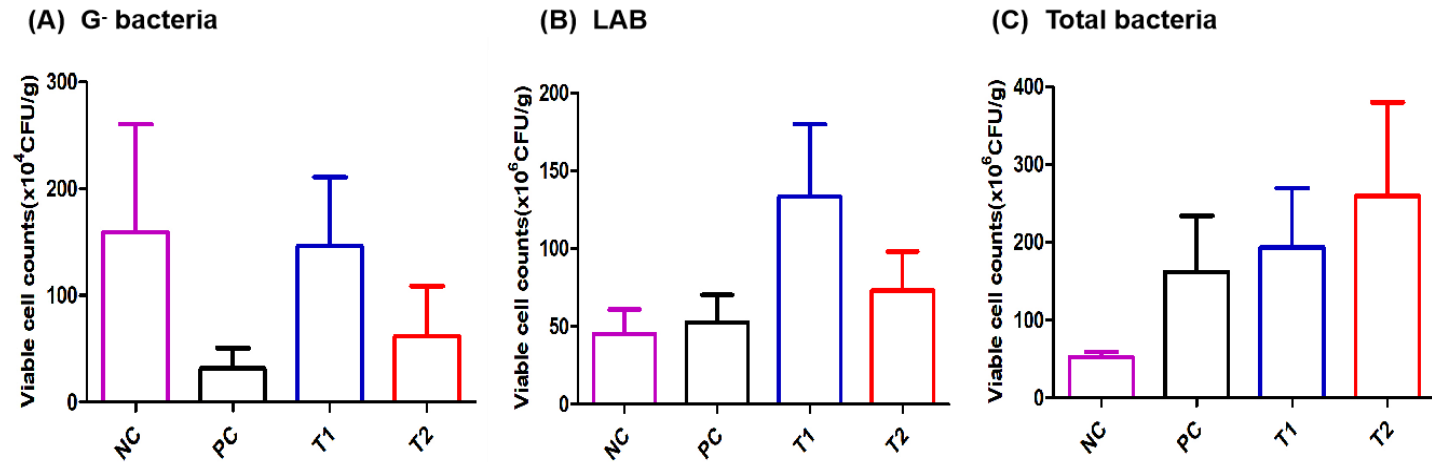
Figure 33. Fecal bacterial content after treatment for five weeks.

(A-C) The viable cell counts of G- bacteria, lactic acid bacteria and total bacteria in fecal samples after five weeks of treatment.

The results of comparison between different treatment groups of fecal samples in the fifth week showed that Gram-negative bacteria in the treatment groups which were treated with probiotics were lower than those in the NC negative control group. And the number of LAB (lactic acid bacteria) and total bacteria also increased in the probiotics treatment group. This suggests that *Lactobacillus plantarum*, as an antibiotic substitute feed additive, can not only inhibit the reproduction of intestinal pathogenic bacteria, but also help to maintain the diversity of intestinal bacteria and maintain the balance of intestinal metabolism.

The results of qPCR showed that the content of *Lactobacillus spp.* in NC, PC treatment group decreased (Figure34A). On the opposite side, T1 and T2 treatment group showed an increasing trend when compared with 5 weeks group. Although *Lactobacillus plantarum* increased in all of the groups, T1 and T2 increased more than those in NC treatment group (Figure34 B). The increase of *Lactobacillus plantarum* in feces of PC group after the addition of antibiotics may be due to the fact that colistin, an antibiotic used in feeding experiments, has a stronger inhibitory effect on Gram-negative bacteria, so it did not affect the reproduction of *Lactobacillus plantarum* in the intestinal tract too much. It

can be seen from the (Figure34 C, D) that the contents of *E.coli* and ETEC in PC group decreased significantly after antibiotic treatment. Although the lactic acid bacteria treatment groups T1 and T2 were not as significant as PC, the overall pathogenic bacteria content was more reduced compared to NC. A greater reduction in pathogenic bacteria in the T2 treatment group compared to the T1 treatment group also confirmed the stronger antibacterial ability of LP-GS1 compared to the wild type.
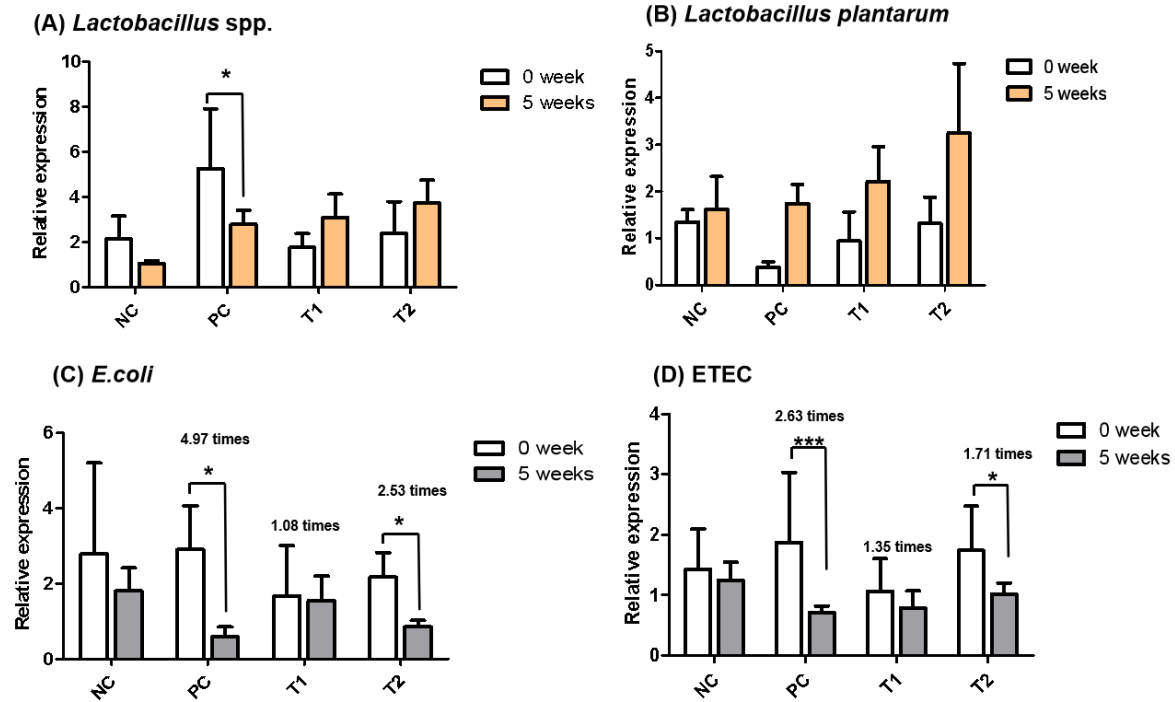
Figure 34. Target bacterial abundance between groups. Changes of (A) *Lactobacillus spp.,* (B) *Lactobacillus plantarum,*

(C) *E.coli* and (D) ETEC in 0 and 5 weeks in different treatment groups.
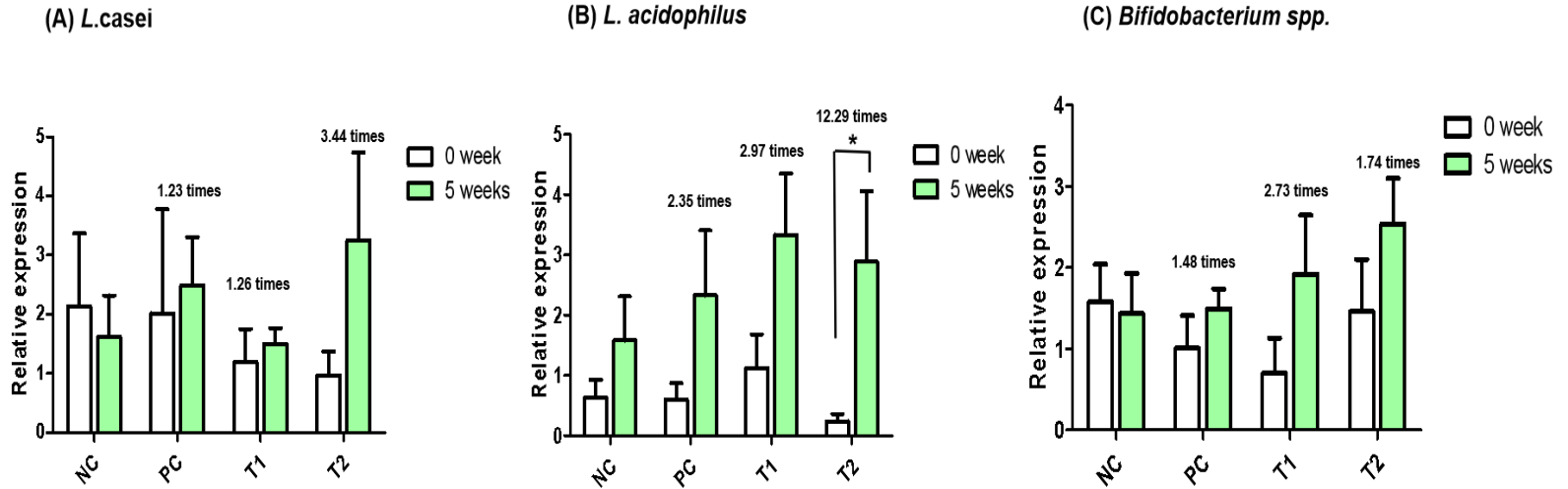
Figure 35. Analysis of the content of other representative beneficial bacteria in feces. Changes of (A) *Lactobacillus casei,*

(B) *Lactobacillus acidophilus,* (C) *Bifidobacterium spp.* in 0 and 5 weeks in different treatment groups.

In addition, because probiotics play an important role in the adjustment of the whole intestinal environment of the host. In order to evaluate the effect of LP-GS1 as probiotics, the changes of *L.casei*, *L.acidophilus* and *Bifidobacterium spp.* in feces were also observed. The results showed that *L.casei* decreased after five weeks in NC group, and increased in the other three groups (Figure 35A). However, T1 increased 1.26 times compared with 0 week, T2 increased 3.44 times compared with 0 week. PC also increased, but the increase was the smallest. Similarly, there was a similar trend in the change of *Bifidobacterium spp.* (Figure 35C). The increase of *L.acidophilus* T2 treatment group was significantly higher than that of the other three groups (Figure 35B). This suggests that LP-GS1, as a probiotics, can not only inhibit the growth of pathogenic bacteria in the intestinal tract, but also help to adjust the intestinal microenvironment and promote the proliferation of other beneficial bacteria.

3) The change of blood metabolites

Blood biochemical can help quickly diagnose the main body system indexes in a short time, and many previous studies have reported the effect of probiotics on the content of components such as TP and BUN in the blood of young piglets (Wang et al. 2017).

The contents of blood components in each treatment group were analyzed every week. The results showed that the content of NEFA (nonesterified fatty acid) in T2 treatment group increased significantly at the fourth week. The concentration of NEFA in serum is related to lipid metabolism, glucose metabolism and endocrine function, and is also needed for lasting activities. The results showed that the enzymes related to energy use in the body of piglets increased significantly after LP-GS1 treatment (Figure 36A), which was more beneficial to the rapid development of the body after weanling. This explained why the T2 treatment group had the largest body weight gain.

BUN (blood urea nitrogen), a nitrogen-containing compound other than protein in the plasma that is filtered from the glomerular and discharged from the body. BUN usually increase when the renal function is not fully decompensated. Therefore, it is used as an index to judge the function of

glomerular filtration in clinic. At the fifth week of the experiment, the content of BUN in T1 treatment group was significantly higher than that in other groups (Figure 36B), indicating that the metabolic function of kidney in T1 treatment group was weaker than that in other groups under the condition of the same protein feeding content.

In addition, according to the statistics in Table 25, there were no statistical differences in other indicators. However, the contents of T-Chol, TG and LDL decreased after probiotics were used instead of antibiotics. This suggests that LP-GS1 had some effect on regulating intestinal health and lowering cholesterol in host and preventing hyperthyroidism.

Table 25. Blood components after 5 weeks.

Parameters	Treatment				*P* value
	NC	PC	T1	T2	
TP, g/dl	6.89±0.79	6.79±0.89	6.42±0.50	6.30±0.53	0.3291
BUN, mg/dl	6.63±1.41	7.22±1.75	9.33±2.49	7.38±2.00	0.0486*
Alb, g/dl	2.74±0.30	2.60±0.37	2.60±0.36	2.46±0.17	0.4470
Glb, g/dl	4.15±0.75	4.19±0.90	3.82±0.34	3.84±0.51	0.5789
A/G ratio	0.68±0.13	0.66±0.18	0.69±0.11	0.65±0.11	0.9583
Ca, mg/dl	10.64±0.47	11.10±0.62	10.87±0.41	10.76±0.39	0.3112
iP, mg/dl	6.49±0.69	6.27±0.63	6.03±0.51	6.16±0.48	0.5007
Glu, mg/dl	108.9±10.26	106.4±7.83	105.6±11.32	106.4±6.28	0.9106
T-Chol, mg/dl	87.13±13.77	84.78±12.87	86.44±14.95	78.88±13.11	0.6614
NEFA, mmol/l	0.089±0.052	0.064±0.035	0.057±0.014	0.050±0.012	0.3457
TG, mg/dl	79.25±74.76	67.44±36.43	55.89±17.57	45.50±14.65	0.4807
LDL, mg/dl	51.75±10.60	47.11±7.09	50.33±7.71	45.63±7.60	0.4841
HDL, mg/dl	27.75±3.73	30.78±4.47	31.44±4.92	27.89±4.78	0.2734
AST, U/l	52.88±10.80	60.11±11.07	58.56±21.01	77.25±30.83	0.1415
GGT, U/l	31.88±8.02	30.56±8.62	28.67±9.09	28.00±8.06	0.8146
Crea, mg/dl	1.15±0.19	1.21±0.15	1.22±0.22	1.13±0.23	0.7406
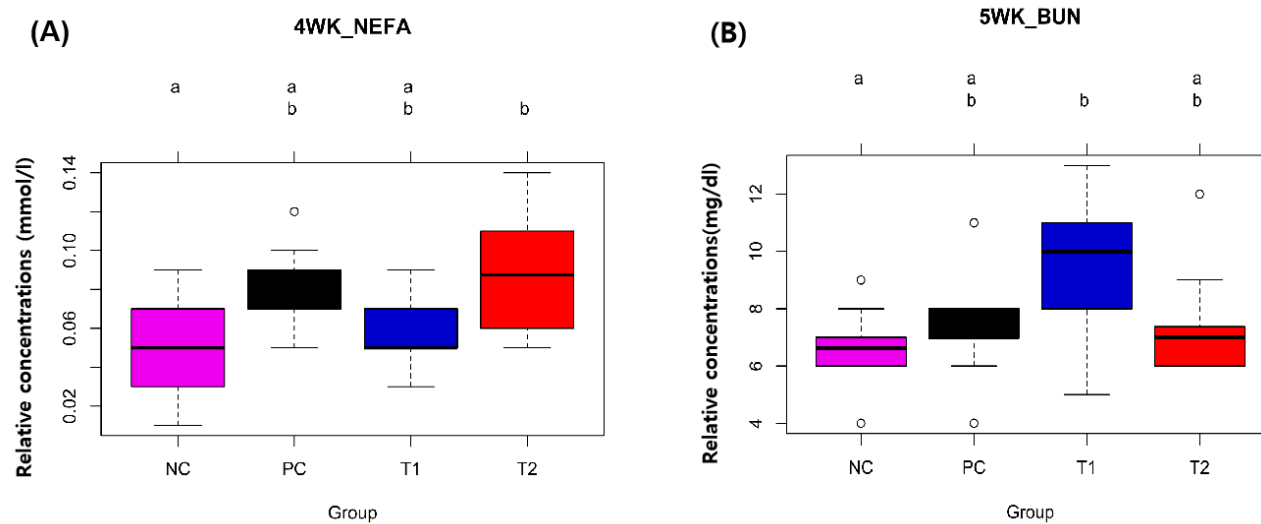
Figure 36. Blood composition change. (A) Comparison of NEFA content in blood of each group after 4 weeks and (B) Comparison of BUN content in blood of each group after five weeks.
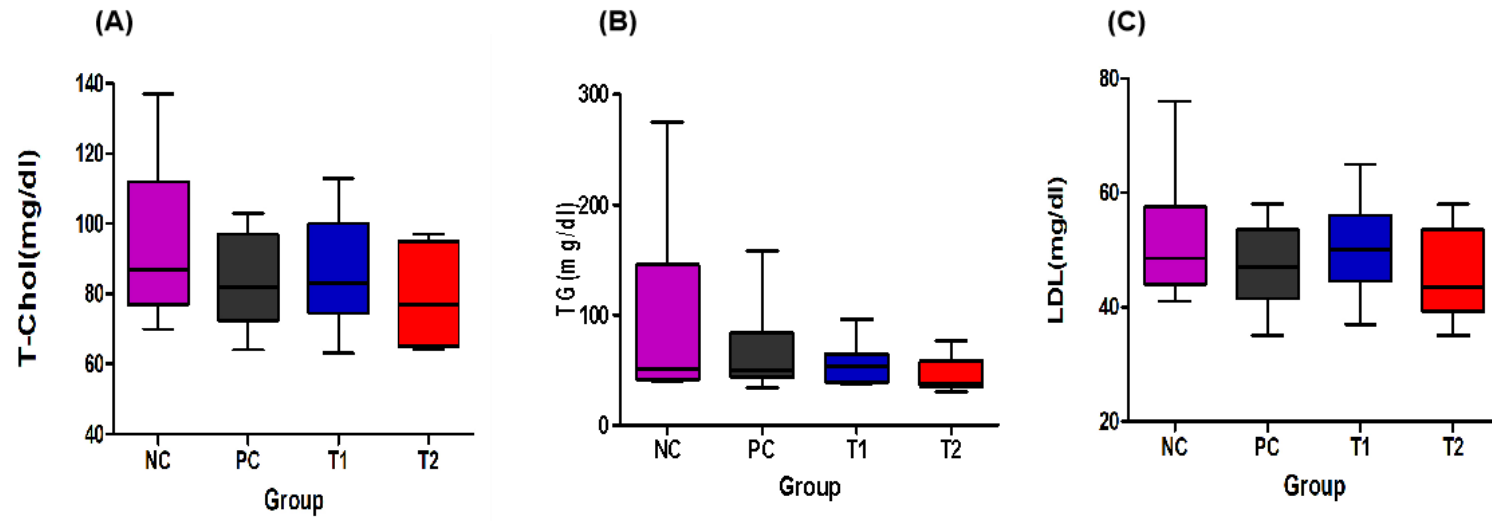
Figure 37. Changes of other blood parameters. (A) Total cholesterol, (B) Triglyceride and (C) Low-density lipoprotein.

4. Discussion

To prevent bacterial diarrhea in weanling piglets and to prove the excellent antibacterial ability of LP-GS1 against *E.coli*, an animal experiment was carried out for five weeks with wild type *Lactobacillus plantarum* as the control.

The results of the growth performance parameters showed that the BW in the T2 treatment group was the highest among the four treatment groups from the second week of the experiment, but there was no significant difference. The incidence of diarrhea in the four groups all increased, and from the 3rd week, the incidence of diarrhea in the PC and T2 treatment groups was significantly lower than that in the NC treatment group. The incidence of diarrhea in the T1 treatment group was also improved compared with the NC treatment group, but there was no significant difference.

On the CFU level with the change in time, the downward trend of the G-bacteria in the PC antibiotic treatment group was the most obvious, followed by the T2 treatment group, while the T1 treatment group only showed a slight decrease. These results suggest that the addition of LP-GS1 can reduce the number of pathogenic bacteria in the intestinal tract. Similarly, using qPCR, we confirmed that LP-GS1 can effectively increase the number of beneficial bacteria including *Lactobacillus spp., L.casei, L.acidophilus* and

Bifidobacterium spp. and reduce the number of enterotoxigenic *Escherichia coli.*

This result shows that LP-GS1 had a better antibacterial effect than that of LP 177 when used as a feed additive. And as a probiotic, it had a better effect on promoting growth compared to the wild type strain.

The results of the blood analysis also showed that lactic acid bacteria, as a probiotic bacteria, not only helped to regulate the intestinal health of the host but also had an effect on reducing cholesterol and on protecting cardiovascular and cerebrovascular health.

The potential of LP-GS1 as an antibiotic alternative was demonstrated by animal feeding experiments, and its effects on the host's intestinal environment as well as the overall health were also verified.

Overall Conclusion

Probiotics are considered to be alternatives to antibiotics because of their ability to regulate intestinal flora and provide health benefits to their hosts. Moreover, with the increasing acceptance of the concept of probiotics, its marketization has increased. In recent years, the development of edible probiotics has expanded from simply eating fermented foods to ingesting specific strains or strains as dietary supplements in the form of tablets or capsules. The expansion of this market requires mature large-scale cultivation and freeze-drying techniques for lactic acid bacteria to store these products for a long time. The purpose of this study was to develop the optimum growth medium and culture conditions using RSM (response surface methodology), which is one of the most commonly used techniques for industrialization. A suitable mixture ratio of CPAs (cryoprotectants) and *Lactobacillus plantarum* (LP-GS1) was also studied, and the effect of LP-GS1 as an antibiotic substitute on the growth of livestock was evaluated.

Response Surface Methodology (RSM) is a statistical method for solving multivariate problems by using a reasonable experimental design method and obtaining certain data. It has been widely used in many application fields. Central composite design provided an efficient and rapid method for screening culture components and culture conditions with the least number of experiments.

In study1, the results showed that the temperature and inoculation amount of molasses in the medium composition had a great influence on the biomass of *Lactobcillus plantarum* GS1. A model for optimizing biomass was established on CCD. The verification of this model at its central point shows that the biomass produced by the batch culture of the microorganism will reach the highest level, which is about 4.75×10^9 cfu/ml under the maximum conditions. This is an optimization method that can reduce the production cost at the same time.

Skim milk, trehalose and MSG have an important role in maintaining the vitality of LP-GS1 during freeze-drying and room temperature storage so that it can be preserved for a long time. Although cryopreservation agents have a certain protective effect on microorganisms in the process of freeze-drying, the killing mechanism related to freezing and storage and the mechanism of protecting microorganisms from injury are complex. Our results could not fully explain the exact mode of action of each agent, but there is no doubt that the proper selection of protective agents as additives in the drying media is a necessary condition for providing strong protection in the storage process of drying laboratories. Several compounds added to a microbial suspension before drying will affect the stability of cells during long-term storage, so it is suggested that they should be used in the mass production.

Animal food safety has become one of the hot topics in the world. Antibiotic-resistant bacteria cause more difficulties in medical and veterinary

practice. Diarrhea in piglets after weanling is a worldwide problem. As a result, probiotics are a promising alternative to antibiotics.

The results of study 2 showed that, as a growth promoter, LP-GS1 can prevent the diarrhea caused by *E. coli* and improve the production performance of the piglets after weaning. The analysis of microorganisms in feces showed that after taking probiotics, the pathogenic bacteria in the intestine of piglets decreased and the beneficial bacteria increased, which indicated that after colonization in the intestinal tract, lactic acid bacteria was beneficial to defend against pathogenic infection and maintain the health of the host intestinal tract. The analysis of blood suggested that lactic acid bacteria also played a certain role in reducing cholesterol and protecting cardiovascular and cerebrovascular health.

Future studies should focus on understanding the close interactions between the microbial flora, mucous physiology and immunity and determining the synergetic effect of on other probiotics as compound additives. At the same time, further studies should optimize the commercial production technology for LP-GS1.

The potential of LP-GS1 as an antibiotic alternative was demonstrated by animal feeding experiments, and its effects on the host's intestinal environment and overall health were also verified.

Study 1	Study 2
Improvement of production efficiency (OFAT + CCD)	
▪ Optimization of culture medium and production conditions	*In vivo* **feeding experiment**
▪ Achieve low cost mass production	▪ Improvement of the BW and ADG
	▪ Effective reduction of diarrhea and mortality
Guarantee of survival rate (OFAT + PBD + CCD)	▪ Increase of the beneficial bacteria
▪ Selection and optimization of cryoprotectants	▪ Reduction of pathogenic bacteria
▪ Product shelf-life confirmation	▪ Regulation of host energy and lipid metabolism
▪ Maintenance of antibacterial ability after freeze-drying	

As an antibiotic substitute, *LP*-GS1 not only has good growth promotion effect, but also has sufficient possibility to achieve mass production

Future prospects

Development of mixed lactic acid bacteria preparation and study on synergistic effect
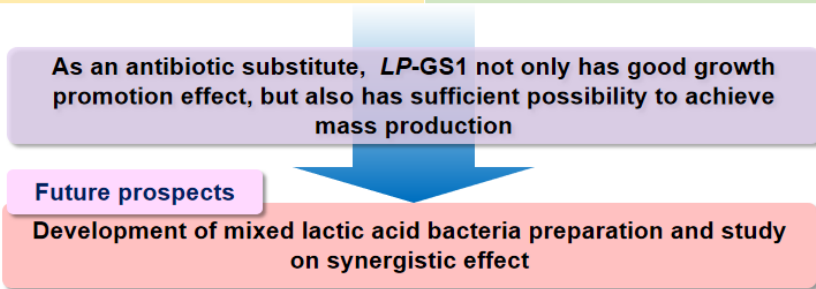
Figure 38. Overall conclusion of the study.

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Summary in Korean

항생제는 일종의 성장촉진제로 1940 년대 이후 가축의 사료효율과 생산성능을 높이기 위해 많이 사용되어 왔다. 그러나 항생제의 남용은 항생제 내성균과 슈퍼박테리아의 출현, 장의 유익균의 손상과 장내균총 불균형 등 여러 부작용을 나타냈다. 이러한 상황의 심각성을 인식하여, 많은 나라들은 축산업에서 항생제의 사용을 금지하였다. 항생제 첨가가 금지됨에 따라 동물사료로 이용 할 수 있는 항생제 대체제의 개발은 현 단계에서 축산업이 시급히 해결해야 할 과제 중 하나이다.

프로바이오틱스는 가축 생산을 개선하기 위한 항생제의 효과적인 대체제로 여겨지고 있다. 프로바이오틱스는 살아있는 생물체로 적절한 양을 섭취하면 숙주의 건강에 이점을 줄 수 있다. 돼지 급성 설사는 새끼돼지가 젖을 뗀 뒤 나타나는 주요 문제점 중 하나로 이 같은 증상은 주로 장독성 대장균 (ETEC)와 장출혈성 대장균 (*Escherichia coli*)이 원인이다. *Lactobacillus plantarum* 은 식품의약 분야에 널리 쓰이는 유익한 유산균으로 대장균과 같은 다양한 그람 음성 병원균에 대한 억제 효과가 있다. 본 연구에서 사용된 *Lactobacillus plantarum* GS1 (LP-GS1)은 유전자 재조합 (genome shulffling) 기술을 이용해 얻은 돌연변이체이다. 이 유전자 재조합 균주는 야생형 *Lactobacillus*

plantarum 177 (LP 177)에 비해 돼지 설사 병원균인 *E.coli* K99에 대해 더 강한 항균능력을 갖고 있다. 따라서 이 유산균의 대량생산을 통해 낮은 가격에 생산을 할 수 있다면 양돈업의 발전에 매우 중요한 의미를 가져올 수 있을 것이다. 그러나 유산균은 성장 조건과 영양 원소에 대한 요구가 상대적으로 까다롭고, 또한 이러한 요구는 종간의 특이성도 가지고 있기 때문에 산업화를 이루려면 이런 문제부터 해결해야 한다.

Study 1은 크게 두 부분으로 나뉜다. Study 1-1에서 LP-GS1의 대량 생산을 실현하기 위해 전체 생산 과정을 최적화했다. 우선 단인소실험법 (OFAT)을 통해 탄소원과 질소원이 발효액 중 세균의 성장에 가장 현저히 영향을 미치는 인자인 것을 확인했다. 각 영양소 간의 상호작용을 동시에 고려하면서 가장 높은 생균수와 낮은 생산원가를 확보하기 위해 반응표면분석법을 사용하여 최적 농도비에 대한 모델링과 예측을 수행하였다. 배지 구성의 최적화를 완료한 후, 생산효율을 한층 더 높이기 위해, 동일한 분석 방법을 사용해 배양 조건 (예: 온도, 접종량, 배양 기간과 초기 pH 등)에 대해 최적화작업을 진행하였다. 최종적으로 LP-GS1의 성장배지는 (탄소원 6.72% 당밀, 질소원 1.21% 효모 추출물)과 성장조건은 (온도 34.85℃, 접종비율

1.29%, 배양시간 20.89hrs, 초기 pH6.73)을 얻을 수 있었으며, 총생균수를 4.75×10^9cfu/ml 얻을 수 있었다. Study1-2 에서는 LP-GS1 의 동결 건조 과정에서의 생존율을 높이기 위해 동결 보호제의 사용을 최적화하였다. 먼저 OFAT 법과 PBD (Planket-Burman Design)법으로 여러 보호제의 보호 효과를 평가한 다음, 선택한 보호제의 최적 배율을 RSM 에 있는 CCD 법으로 예측했다. 이에 최적화된 동결보호제의 조건 (동결보호제 조건)을 이용하여 (동결건조후 생존율)의 동결건조 후 85%이상의 생존율을 얻을 수 있었다. 이로써 study1 에서는 LP-GS1 의 대량생산 조건 최적화를 완료하였다.

Study 2 에서는 *in vivo* 이유자돈 실험을 통해 항생제 대체 사료첨가제로써 LP-GS1 이 사용될 가능성을 검증하였다. 전 실험기간에 걸쳐 성장성적을 확인하였을 때, LP-GS1 을 처리한 그룹이 양성 대조군 (항생제 처리군)과 유사하게 성장에 긍정적인 영향을 준다는 것을 확인할 수 있었다. 둘째주 부터 LP-GS1 처리그룹의 체중 (BW)과 일당증체량 (ADG)이 다른 그룹보다 높게 나왔다. 또한 전체 실험에서 자돈의 설사와 사망의 발생률을 관찰한 결과 프로바이오틱스를 첨가한 그룹에서, 특히 LP-GS1 그룹의 설사 발생률이 대조군보다 유의적으로 감소하는 것을 확인하였다. 자돈 분변 중 미생물의 함량을 생균수와

DNA 수준에서 측정한 결과 음성 대조군에 비해 LP-GS1 처리그룹에서 유익균(*Lactobacillus spp., L.casei, L. acidophilus, Bifidobacterium spp.*)의 함량은 많아졌으며 병원균(*Escherichia coli*)의 함량은 유의적으로 줄어드는 것을 확인할 수 있었다.

Study 1 과 2 의 결과들을 통해 LP-GS1 은 유익한 유산균으로서 비교적 저렴한 가격으로 대량생산이 가능하다는 것을 보여주었으며, 가축의 사료첨가제로 이용하면 장내 균총의 균형을 조절하고 가축 생산성을 높이는 데 도움이 된다는 것을 확인할 수 있었다. 따라서 LP-GS1 은 항생제의 대체제로서 이용가치가 높다는 것을 확인할 수 있었고, 더 다양한 가축에서 이용 가능성을 지니고 있다고 생각한다.

주요어: 락토바실러스 플란타룸 GS1, 대량생산, 최적화, 반응표면분석, 세균성 설사, 이유자돈

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