Master's Thesis of Science in Agriculture

Engineering of Polyphosphate Accumulating Probiotic Strains as Chronic Kidney Disease Treatment

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Engineering of Polyphosphate Accumulating Probiotic Strains as Chronic Kidney Disease Treatment

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Abstract

Cardiovascular disease caused by hyperphosphatemia accounts for 47% of deaths in chronic kidney disease (CKD) patients. Existing treatments to address hyperphosphatemia include dialysis, protein (phosphorus) restriction diet, and phosphate binders. However, there is a concern that it may decrease the life quality of CKD patients and side effects have been reported several times. Probiotics, which is also polyphosphate accumulating organisms (PAOs), is suggested as an alternative with no disadvantages described above. *Lactiplantibacillus paraplantarum* KCCM 11826P strain, which was found to have excellent polyphosphate accumulation ability in previous studies, was further improved with inverse metabolic engineering. First, a safety evaluation was confirmed according to 'Probiotics guidelines'. After resequencing process, it was confirmed that finally selected *L. paraplantarum* SNUP7 strain from *L. paraplantarum* KCCM 11826P mutated *tuf*, *dnaK*, and *groL,* so that absorbed more 18.2% of phosphorus than the *L. paraplantarum* KCCM 11826P in phosphate-rich medium. Even in animal test, CKD rats administered *L. paraplantarum* SNUP7 probiotics (Nx+P) had a 30.5% decrease in blood phosphorus level compared to CKD rats administered vehicle (Nx+V). And kidney injury level in Nx+P group was also suppressed, Nx+V group did not. Therefore, *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 strains act as bio-phosphate binders and have the potential to contribute to alleviating CKD symptoms and improving life quality of CKD patients.

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List of Abbreviations

Chapter 1.

CKD: Chronic kidney disease

GFR: Glomerular filtration rate

KDIGO: The kidney disease improving global outcomes

P_i: Inorganic phosphate

polyP: Polyphosphate

PAOs: Polyphosphate accumulating organisms

IME: Inverse metabolic engineering

CAGR: Compound annual growth rate

Pst: Phosphate-specific transport

PhoR: Phosphate regulon sensor protein PhoR

PhoU: Phosphate-specific transport system accessory protein PhoU

PPK: Polyphosphate kinase

PPX: Exopolyphosphatase

EBPR: Enhanced biological phosphorus removal

Chapter 2.

FAO: Food and agriculture organization of the United Nations

WHO: World health organization

MIC: Minimal inhibition concentration

MRS: de Man, Rogosa and Sharp

SD: Standard deviation

KCCM: Korean culture center of microorganisms

KCTC: Korean collection for type cultures

ATCC: American type culture collection

LarA: DL-lactate racemase

LDH: Lactate dehydrogenase

MFDS: Ministry of food and drug safety

BSH: Bile salt hydrolase

EFSA: European food safety authority

PHAST: Phage search tool

D.W: Distilled water

Chapter 3.

P: Phosphorus

NTG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

FACS: Fluorescence activated cell sorting

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

O.D: Optical density

PPM: Potassium phosphate monobasic

CFU: Colony forming unit

CFS: Cell free supernatant

Nx: 5/6 nephrectomized

NGS: Next generation sequencing

SNPs: Single-nucleotide polymorphisms

EF-Tu: Elongation factor Tu

Discussion

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium

LDH: Lactate dehydrogenase

IS: Indoxyl sulfate

Chapter 1.

Research background

1. Chronic kidney disease

Chronic kidney disease (CKD) is a progressive disorder characterized by structural and functional abnormalities of kidney more than 3 months. On the basis of glomerular filtration rate (GFR) and the degree of albuminuria (typically defined as an albumin-to-creatinine ratio of >30 mg/g), The Kidney Disease Improving Global Outcomes (KDIGO) defines a severity categorization, establishing a number of stages of CKD (Murton et al., 2021) (Table 1.). Because GFR is a recognized indicator of renal excretory function and albuminuria is a sign of renal barrier dysfunction (glomerular injury). These two factors are utilized to categorize CKD. Both have been shown to be accurate indicators of the progression of CKD. The number of patients with CKD has also been rising; in 2017, 843.6 million people were thought to be affected globally (Jager et al., 2019). Diabetes mellitus and hypertension are the two underlying conditions that cause CKD the most frequently (Romagnani et al., 2017). This is because high blood sugar concentration and high blood pressure affect capillaries and nephrons of the kidney. CKD continues to have a phenomenon in which waste excretion is not carried out smoothly, leading to various complications. The most lethal complication is cardiovascular disease.

Table 1. Prognosis of CKD by GFR and albuminuria categories Red indicates a very high hazard of illness progression; orange indicates a high hazard of illness progression; yellow indicates a moderately hazard of illness progression; green indicates low hazard of illness progression

			Persistent albuminuria categories		
			A1	**A2**	**A3**
			Normal to mildly increased	Moderately increased	Severely increased
			<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min/1.73 m^2)	**G1**	Normal or high	>90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

2. Mineral bone disorder in CKD

2.1. Hyperphosphatemia

Hyperphosphatemia is a mineral bone disease in which the level of phosphorus in the blood higher than normal. This disease is diagnosed when its level is above 4.5 mg/dL. One of the major causes of the disease is CKD. Normal adults consume 1000-1200 mg of phosphorus per a day. Through absorption of phosphorus in the intestine, a net weight of about 800 mg enters exchangeable phosphorus pool in the body. The level of phosphorus excessively accumulated in this pool is controlled to normal levels through skeletal deposition (300 mg/day), intestinal secretion (400 mg/day), and renal excretion (400 mg/day). In CKD patients, loss of phosphorus homeostasis is occurred due to renal excretion failure. The phosphorus concentration in the exchangeable phosphorus pool is not properly regulated, which accumulates phosphorus in the blood and causes hyperphosphatemia (Fig. 1.). Excessive phosphorus reacts with surrounding calcium ions and forms calcium phosphate. This is called metastatic calcification, and it usually occurs in the cardiovascular system. The calcium phosphate hardens blood vessels and causes arteriosclerosis. As a result, blood vessel damage becomes severe, leading to cardiovascular disease (Askar, 2015). About 47 % of CKD patients' deaths are due to this cardiovascular disease in Korea (Hong et al., 2021).

2.2. Treatments

The current KDIGO clinical practice guidelines suggest limiting dietary phosphorus consumption, increasing dialysis clearance, and using phosphate binders to bring increased blood phosphorus concentration in CKD patients on

dialysis back into normal range (Isakova et al., 2017). Such dietary management appears to have several advantages, including reducing the buildup of nitrogenous wastes and decreasing the metabolic abnormalities typical of severe CKD. But this approach can lead to protein energy malnutrition and cause a decrease in muscle mass in the body (Ikizler, 2009). Numerous previous publications have noted the fundamentally challenging stoichiometry of phosphorus removal by dialysis. Phosphorus elimination during hemodialysis administered three times per week ranges from 800-1200 mg/session or 2.4-3.6 g/week, depending on the effectiveness of the dialysis method and the average blood phosphorus level during dialysis. However, the average phosphorus consumption is between 0.8-2.0 g/day, or 6-14 g/week (Daugirdas, 2015). In addition, dialysis can only be performed in the presence of professional equipment and personnel. CKD patients who have to undergo frequent dialysis should only stay near the facility. This becomes a factor that lowers quality of life of the patient. The administration of phosphate binders is nearly always necessary with a regular diet and traditional three times per week dialysis regimens. Phosphate binders come in three broad categories: calcium- and aluminum-based binders, and the new non-calcium-based binders. Calcium-based binders are inexpensive and have been widely used. However, it causes hypercalcemia, causing gastrointestinal disorders. It can also accelerate vascular calcification (Hutchison, 2009). Aluminum-based binders are also cheap but can cause aluminum poisoning and increase the risk of dementia, osteomalacia, and anemia (Group, 2009). Sevelamer has less risk of these but has a lower phosphate binding capacity than other phosphate binders. However, sevelamer is expensive and its pill burden is high because CKD patients have to swallow the pill with a lot of frequency (Chan et al., 2017). Lanthanum is a trivalent metal phosphate binder, showing a phosphate affinity similar to that of an aluminum-based binder (Finn, 2006). It is in the form of powder, so lanthanum reduces the pill burden than chewable tablets (Sakurada et al., 2013). However, it is as expensive as sevelamer, and lanthanum can accumulate in the liver and bone (Zhang et al., 2013). And it can be accompanied by gastrointestinal intolerance. Sucroferric oxyhydroxide is actionable at a wide range of pH, with a peak at pH 2.5. This characteristic enables a smooth phosphate binding mechanism even in the strongly acidic environment. However, it is expensive and can cause

gastrointestinal side effects (Chan et al., 2017) (Table 2.).

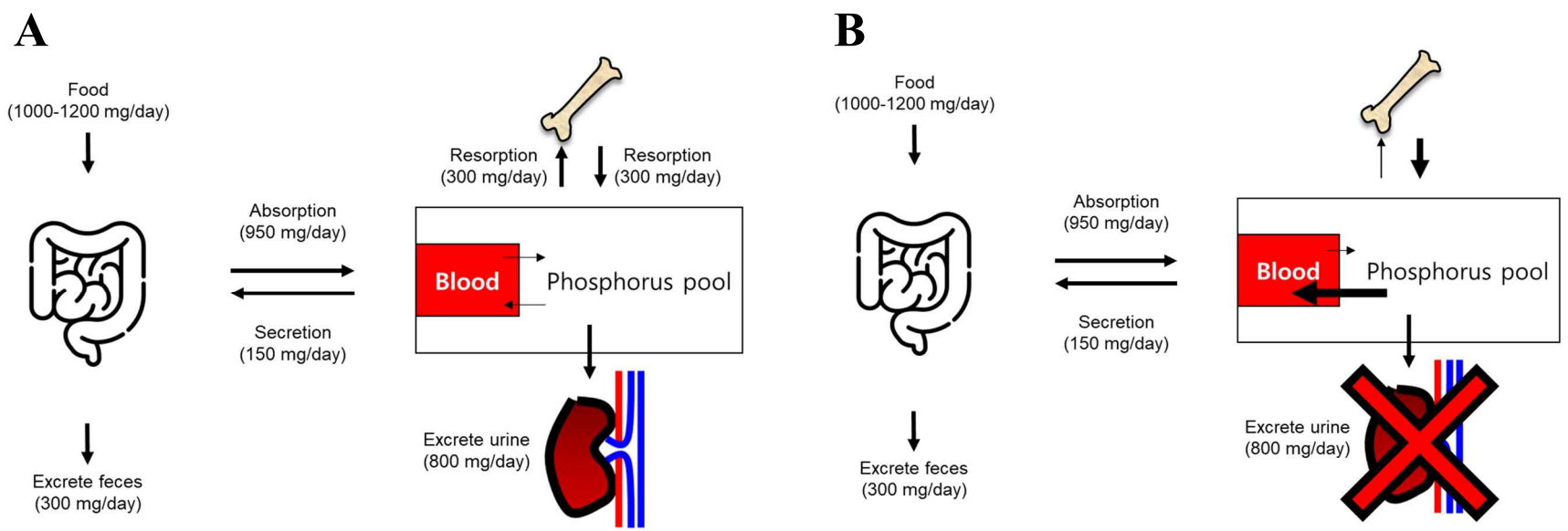


Fig. 1.1. Phosphorus homeostasis in the body (A) normal physiology (B) CKD patients

Table 2. Characteristics of phosphate binders (Chan et al., 2017)

Based on	Example	Mechanism	Advantages	Disadvantages
Aluminum based	Aluminum hydroxide	Generates of insoluble phosphate complexes in the intestine	Cheap, calcium-free, capable of binding phosphate across a wide pH range	No safe dose has been identified, there are severe side effects (such as possible central nervous system toxicity, microcytic anemia, osteomalacia, and gastrointestinal distress), and serum aluminum levels need to be monitored.
Calcium based	Calcium carbonate	Generates insoluble phosphate complexes in the intestine	Somewhat efficient, reasonably priced	Vascular calcification, the need for high doses to be effective, and unpleasant
Non-calcium based	Sevelamer hydrochloride	An anion exchange resin	Calcium-free, reduction of lipids	Pricey, high pill burden, gastrointestinal side effects
	Lanthanum carbonate	Creates insoluble phosphate complexes in the intestine	Low pill burden, good effectiveness, operates in wide range of pH, no adverse effects on bone histology	Pricey, gastrointestinal side effects, indefinite long-term effects
Iron based	Sucroferric oxyhydroxide	An iron-based ligand exchange molecule	Low pill burden, operates in wide range of pH, negligible systemic absorption	Pricey , gastrointestinal side effects

3. Polyphosphate accumulating organisms (PAOs)

Phosphate is considered an essential component of all living things. Many microorganisms accumulate the surrounding inorganic phosphate (P_i) and store it in the form of polyphosphate (polyP) (Kulaev, 1987; Kulakovskaya et al., 2014). Intracellular polyphosphate helps many physiological activities associated with survival in organisms (Kornberg et al., 1999). Polyphosphate accumulating organisms (PAOs) are specific microbial groups that could absorb and accumulate phosphorus into cells in a phosphorus-rich environment (Fig. 2.). This group has been covered a lot in the field of wastewater treatment for enhancing biological phosphorus removal (EBPR). *Acinetobacter* spp. turns out to be the most powerful PAOs in EBPR (Fuhs & Chen, 1975). Recently, *Serratia marcescens* has also shown the potential to remove phosphate from wastewater (Chaudhry & Nautiyal, 2011). However, the PAOs described above are not safe for human consumption. Therefore, they are not suitable for use as a probiotics (Anand & Aoyagi, 2019). For the synthesis of polyP, PAOs use polyphosphate kinase (PPK). And for the degrading polyP, exopolyphosphatase (PPX) is used. In addition, factors such as the Pst system for the transport of phosphate and the PhoR and PhoU required for transport signal transmission are believed to affect polyphosphate accumulation (Moon et al., 2022). Some studies have been conducted on the accumulation of polyP in PAOs, however it is not fully understood (Bowlin & Gray, 2021).

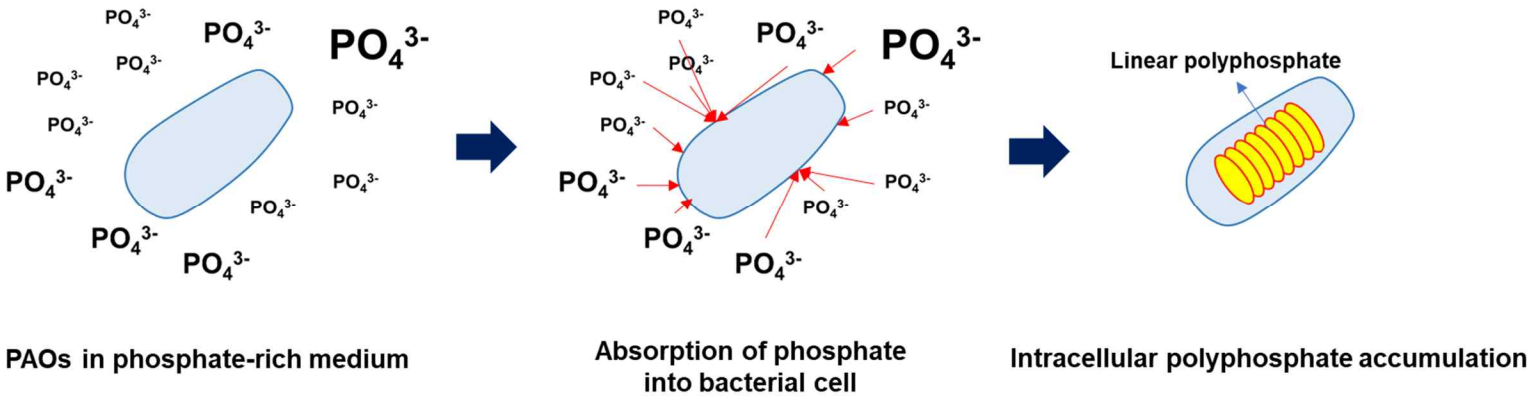


Fig. 1.2. Overview of phosphate accumulation in PAOs

4. Inverse metabolic engineering

To successfully utilize metabolic engineering techniques, widespread knowledge of a clear biochemical pathway is especially important. Efficient production of a specific metabolites or cell phenotype is based on this knowledge. However, a lot of cellular features that are beneficial to industries and research, are either poorly understood or still unknown (Dasgupta et al., 2020). To solve this problem, inverse metabolic engineering (IME) is suggested. IME is useful strategy to determine the secrets of cell factories. This approach includes the followings: (i) random mutagenesis using chemical and physical materials on the wild type strain, (ii) adaptive evolution by serial batch culture, (iii) mutant selection which has desired phenotype using high-throughput screening, (iv) genome sequencing of screened mutant and detecting genetic variations which effects that phenotype, (v) drawing possible metabolic pathway and endowing genetic manipulation on host strain. (Bailey et al., 1996; Winkler & Kao, 2014) (Fig. 3.). Random mutagenesis is a priority step in this strategy since many organisms normally have low inherent mutation rates. This method is used to increase the genetic multiplicity and essential mutations. And accumulation of successive mutation can be controlled by adaptive laboratory evolution. Bioreactors could perform an important role in this process by giving controllable culture conditions such as pH, nutrient, temperature, and dissolved oxygen (Zhu et al., 2018). To screen ideal mutant, high-throughput screening is a key factor. Two main approaches of this method are screenings based on growth and color/fluorescence. Environmental and chemical tolerance strains are typically screened using a growth-based screening (Zhang et al., 2012). For yield-related screening, color/fluorescence-based screening is typically utilized. Additionally, color or fluorescence could be created by reacting products with exogenous reagents or enzymes (Reyes et al., 2014). By sequencing of the genome in the screened mutant, gene variations that affect beneficial phenotype would be revealed. After investigating feasible pathways, the gene variations would be applied by direct genetic engineering to host strain.

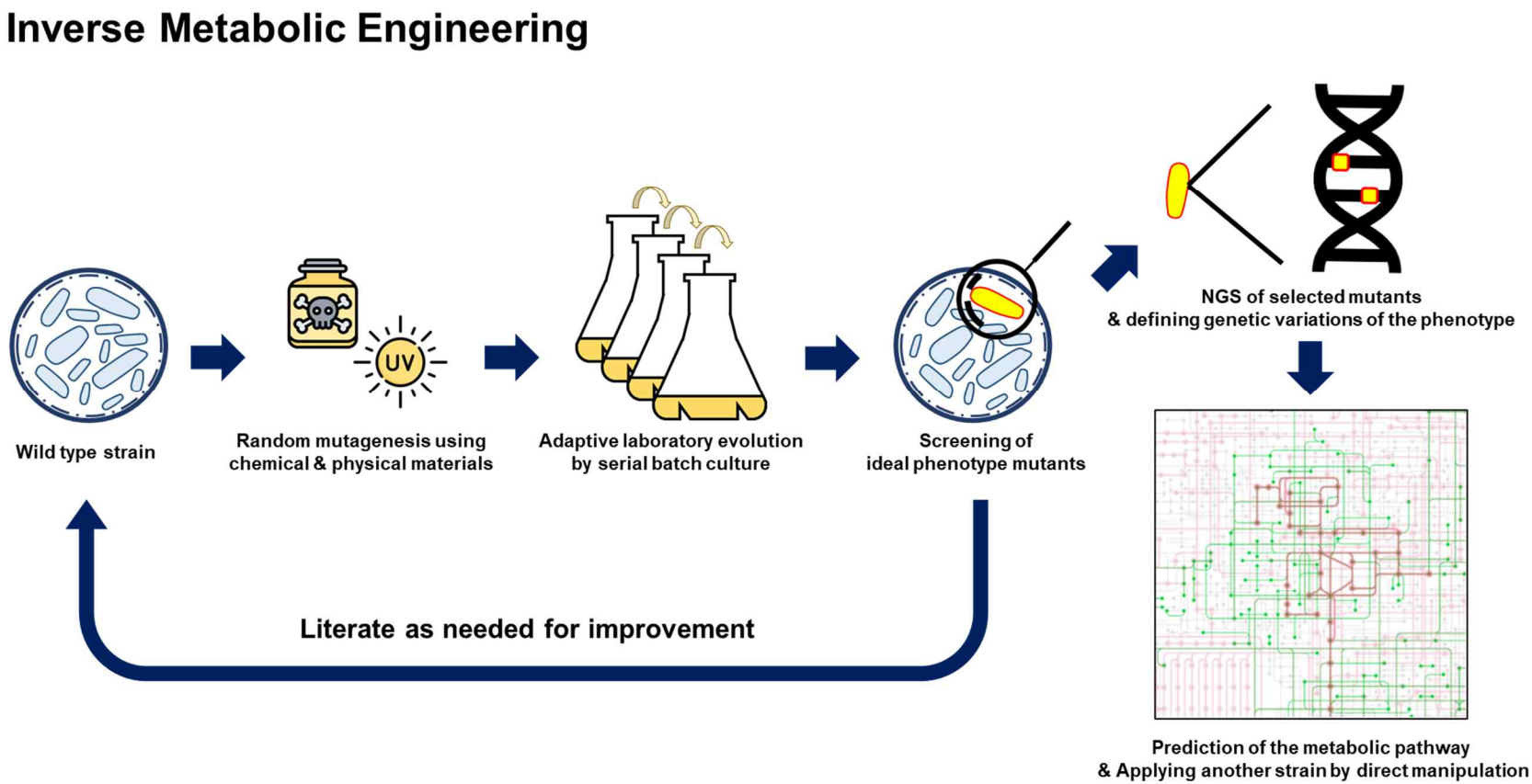


Fig. 1.3. Overview of inverse metabolic engineering

5. Chemical mutagenesis

Microorganisms, which have new genetic characteristics and ideal phenotypes, are often created by repeatedly performing spontaneous mutation and selection. In this classical strain improvement, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) has been utilized as a common mutagen to create mutants that show better output (Ohnishi et al., 2008). NTG works by attaching alkyl groups to the O^6 of guanine and O^4 of thymine, which could result in mutations that switch between GC and AT (Gordon et al., 1990). By producing methylated bases in DNA, this causes incorrect corporation during the following cycle of DNA replication (Ito et al., 1994).

6. Probiotics

Probiotics is defined as living biological agents that could provide health benefits to host when consumed in an appropriate amount. And this word also implies ‘for life’ in Greek (FAO, 2002). In recent years, probiotic strains could be consumed as a preventative measure to preserve the equilibrium of the intestinal microflora and so improve the ‘well-being’ of both humans and animals. A more reasonable view that incorporates knowledge of the beneficial effects of bacteria in human health has emerged because of increased public awareness of related research (Cunningham et al., 2021). As a result of these developments, probiotics is becoming more widely known and accepted in a familiar way. From 2021 to 2030, the probiotics market is projected to increase at a compound annual growth rate (CAGR) of 7.5 %, from a market size of USD 58.17 billion in 2021 (Research, 2022). Abundant microbial species have been used as probiotics such as yeast, lactic acid bacteria. These microorganisms could stimulate and modulate immune response. And probiotic microbes could normalize intestinal microflora by colonization resistance which means competing for nutrients and location. The final process also has metabolic consequences such as bile salt deconjugation and secretion of various metabolites, the hydrolysis of lactose, the decrease of mutagenic and toxic events in the gut, and the provision of nutrients to the colon epithelium (Abatenh et al., 2018; Jain & Sharma, 2012). Combining these intestinal metabolic characteristics with the probiotic strains, probiotics has the ability of the alleviation of chronic disease.

7. Overall objectives

CKD is one of the diseases that is on the rise around the world due to a modern diet with high sugar and fat. When the kidney, which is responsible for the control of waste and minerals in the body, breaks down, the substances accumulate in the body. And this situation causes a bad prognosis. Among minerals, excessive phosphorus can cause cardiovascular disease through metastatic calcification. Existing treatments for hyperphosphatemia caused by this include dialysis, phosphate binders, and protein-restricted diet. However, these reduce the life quality of the CKD patients. In the case of phosphate binders there are side effects that adversely affect physiological activity in the body.

To supplement these points, an alternative approach called probiotics is proposed. The proposed *Lactiplantibacillus paraplantarum* KCCM 11826P as a probiotic strain has been proven to be PAOs through previous studies. Using this characteristics, therapeutic probiotics with the possibility of reducing the blood phosphorus level of CKD patients were developed.

Therefore, this study focused on the suitability as safe probiotics and inverse metabolic engineering of *L. paraplantarum* KCCM 11826P.

In Chapter 2, several tests were run to confirm the strain's safety when ingested in accordance with FAO/WHO probiotics guidelines. Antibiotic resistance and hemolysis test were performed. Acid and bile salts tolerance tests were carried out to determine the viability when delivered into a human gastrointestinal tract. Moreover, gene annotation was accomplished through whole genome sequencing using the HiSeqXten platform and used for investigating existence of toxic metabolite genes.

In Chapter 3, inverse metabolic engineering was performed to identify the genes that affect polyphosphate accumulation ability. First, a toxic chemical was treated in *L. paraplantarum* KCCM 11826P to induce random mutagenesis. FACs technology was then used to select the group that accumulates the most polyphosphate among than other mutant groups. With 300 single colonies in this group, color and fluorescence-based screening methods to determine which candidate strains consumed more

phosphate and absorbed it into their cells. The finally selected mutant strain was confirmed through whole genome sequencing what genetic variations occurred compared to wild type strain. Animal test using CKD rats were conducted to determine whether *in vitro* as well as *in vivo* showed the same tendency and alleviated kidney injury.

8. Significance of research

By conducting a safety evaluation of *L. paraplantarum* KCCM 11826P, it would be possible to provide a clear basis for whether *L. paraplantarum* KCCM 11826P could be used as a probiotics. In addition, IME would secure improved strains with increased phosphorus absorption capacity over wild type strain, while at the same time confirming which genes affect this phenotype. If the genes are revealed, this result could provide promising directions for the molecular biological mechanisms of PAOs. And from a clinical standpoint, if we apply that genetic facts to produce probiotic products with much more ideal phenotypic mutant strains, we might offer a better future for CKD patients and their families.

Chapter 2.

Characteristics of *Lactiplantibacillus paraplantarum* KCCM 11826P according to 'Probiotics guidelines'

1. Introduction

There is a broad scientific agreement that a preliminary *in vitro* evaluation must be conducted in accordance with the 'Probiotics guidelines' from FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization) (FAO/WHO, 2002) in order to evaluate the qualities of probiotic bacterial strains. A scheme outlining these guidelines for the evaluation of probiotics is specified in the following; (i) strain identification, (ii) *in vitro* tests to assess functional characterization, (iii) *in vitro* or *in vivo* tests for safety assessment, and (iv) Health claims and labeling. In this study, the isolated strain from Korean traditional fermented food was judged in accordance with 'Probiotics guidelines' from FAO/WHO and MFDS (Ministry of Food and Drug Safety) in Korea and the types of tests are as follows (FAO/WHO, 2002); (i) EFSA MIC cut-off of antibiotics, (ii) hemolysis, (iii) acid and bile salt tolerance, (iv) metabolic properties, and (v) toxin production status. Additionally, there are cytotoxicity tests such as LDH assay, and *in vivo* tests, but these tests are excluded. Since they are unannounced strains with culinary experience that meet the third standard of the MFDS criteria.

2. Materials & Methods

2.1. EFSA MIC cut-off value of antibiotics

As previously described, the MIC (Minimum Inhibition Concentration) is the lowest concentration of an antimicrobial drug that will impair a microorganism's ability to grow visibly after overnight incubation against indicator strains. In this study, the MIC test was performed to verify the minimum inhibition concentration required for antibiotics to inhibit *L. paraplantarum* KCCM 11826P strain. Ampicillin, kanamycin, and streptomycin were used in this experiment. These antibiotics' MIC cut-off value (mg/L) was quoted by EFSA (European Food Safety Authority). All these antibiotics were started at a concentration of 0.2 mg/ml, and a 2-fold dilution process was repeated 10 times to reach a concentration of 0.1 %. $O.D_{600}$ was determined for 24 h using microplate reader. Each well included a mixture of 100 μl of medium + *L. paraplantarum* KCCM 11826P and 100 μl of antibiotics dissolved in suitable solvent. *L. paraplantarum* KCCM 11826P was inoculated into each well at the concentration of 5×10^6 CFU/ml.

2.2. Hemolysis

To decide whether *L. paraplantarum* KCCM 11826P cause hemolysis, hemolysis test was conducted. *L. paraplantarum* KCCM 11826P was cultured for 24 h on 5 % sheep blood agar plate (Bandio, Pocheon, Korea). *Streptococcus mutans* and *Staphylococcus aureus* were both cultivated as the α- and β-hemolysis control respectively. Hemolysis activity of *Enterococcus faecium* was also observed as the γ-hemolysis. The streaking process of control strains was also performed under identical circumstances. To observe the hemolytic effect on the blood agar plate, the plate was held up to the light source. And comparison with three different hemolysis controls was also carried out.

2.3. Cell viability test

2.3.1. Acid tolerance test

To determine whether *L. paraplantarum* KCCM 11826P has resistance under a gastric environment which is high-acidic, acid tolerance test was implemented. For this test, the method of Somashekaraiah was cited with slight variation (Somashekaraiah et al., 2019). The strains were cultivated in MRS broth at 30 ℃. In tubes containing 5 ml of MRS broth that had been pH-adjusted with 1 N HCl, 7 x 10^8 CFU/ml of inoculant was added. The pH values were 2, 3, and 4. Each tube was incubated for 0, 2, and 4 h. Cell viability was determined by counting CFU on the MRS agar plate at each incubation time.

2.3.2. Bile tolerance test

To validate whether *L. paraplantarum* KCCM 11826P has resistance to bile salts, the methods of Singhal was performed with slight modifications (Singhal et al., 2021). The culture was cultivated in MRS broth at 30 ℃. 7 × 10^8 CFU/ml of inoculant was added into tubes containing 5 ml of MRS broth adjusted with various bile salt concentrations using bile salts. The concentrations of bile salts were 0.1 and 0.3% (w/v). Each tube was incubated for 0, 2, and 4 h. Cell viability was determined by counting CFU on the MRS agar plate at each incubation time.

By counting the surviving cells (N_1) on the MRS agar plates in comparison to the initial cell counts (N_0) before to incubation, the survival rate (%) of the strain exposed with acid and bile salts was determined as follows.

$$\text{Survival rate (\%)} = \frac{\log N_1}{\log N_0} \times 100$$

2.4. Metabolic properties

The metabolic properties of *L. paraplantarum* KCCM 11826P were tested to establish whether there is a potential risk. The investigations consist of D-lactate production and bile salt hydrolase activity. L-lactate is metabolized in humans, but isomer D-lactate is not. Therefore, the probiotic strains with DL-lactate racemase can cause acidosis in newborns, children, and patients with short bowel syndrome when its activity is high. Bile salt hydrolase (BSH) can increase intestinal settlement by protecting the probiotic strains from bile salt. But it may be challenging to decompose fatty acids due to the deconjugation of bile salt by BSH. The complete genome sequence of *L. paraplantarum* KCCM 11826P was analyzed to confirm the presence of genes coding these enzymes using Geneious 6.0.6. Although the guidelines suggest experimental methods on the degree of bile salt deconjugation activity, this is not legally effective and is stated to be replaceable when there is a verification method equivalent thereto. *L. paraplantarum* KCCM 11826P, *L. plantarum* WCFS1, *L. plantarum* KCTC 3108, and *L. casei* ATCC 393 were cultured in MRS broth for 24 h to quantification D-lactate. After incubation for 24 h, cell-free supernatants (CFS) were collected. D-lactate level in each CFS was analyzed using D-lactate assay kit (Megazyme International, Wicklow, Ireland). This analysis was carried out according to the manufacturer's instructions.

2.5. Toxic metabolites

FAO and WHO insist that a minimal safety evaluation is required, which includes investigation of specific metabolites productions such as toxin. Examples of these are cereulide synthetase, non-hemolytic enterotoxin, hemolysin BL, cytotoxin, histamine decarboxylase, and tyramine decarboxylase, etc. These metabolites can induce pathogenesis such as organ failure, diarrhea, and any other pain in humans.

The complete genome sequence of *L. paraplantarum* KCCM 11826P was analyzed to confirm the presence of genes related to toxin production using Geneious 6.0.6.

3. Results

3.1. EFSA MIC cut-off value of antibiotics

MIC test is an experiment to check antibiotic resistance. When MIC cut-off value is higher than the standard, it is deemed to exhibit antibiotic resistance. Since more antibiotics must be used to suppress the corresponding bacteria. Alternatively, if it is lower than the standard, it is determined that there is no resistance and the probiotic standard could be met. The criteria for *Lactobacillus* facultative heterofermentative to which *L. paraplantarum* KCCM 11826P belongs in accordance with EFSA are listed in Table 3. Ampicillin met the criteria, but kanamycin and streptomycin exceeded the criteria. It was necessary to confirm whether kanamycin and streptomycin resistance were acquired or intrinsic. If these are acquired resistance, *L. paraplantarum* KCCM 11826P is impossible to use as probiotics because the characteristics could be transmitted to the harmful bacteria in intestine. To confirm this, PHAge search tool (PHAST) and Mobile Element Finder tools were used and results shown in Table 4. It was confirmed that kanamycin and streptomycin resistance are not acquired but intrinsic properties of *L. paraplantarum* KCCM 11826P (Guo et al., 2017; Ouoba et al., 2008). There have been numerous reports of antibiotic resistant characteristics in *Lacobacilli* (Campedelli et al., 2019). And the majority of *Lactobacillus* species are naturally resistant to aminoglycosides, including kanamycin and streptomycin (Abriouel et al., 2015). In *L. paraplantarum* KCCM 11826P, there are two genes that *lmrA* and *bmrA* gene. These genes produce the factors that make up multidrug resistance ABC transporter. ABC transporters cause drug resistance and a limited bioavailability of drugs by pumping a variety of drugs out of the bacterial cells with ATP hydrolysis (Choi, 2005).

Table 3. EFSA MIC cut-off value of antibiotics against *L. paraplantarum* KCCM 11826P

Antibiotics	MIC cut-off value (mg/L)	
	In this study	EFSA, 2012
	L. paraplantarum KCCM 11826P	*Lactobacillus* facultative heterofermentative
Ampicillin	0.78	4.00
Kanamycin	10,000	64.00
Streptomycin	2,500	64.00

Table 4. The presence of acquired antibiotic resistance-related genes in *L. paraplantarum* KCCM 11826P

(–) means that there is no corresponding gene.

Antibiotics	Target Gene	Ref.	*L. paraplantarum* KCCM 11826P	
			Chromosome	Plasmid
Kanamycin	*aph-III*	Guo et al., 2017	-	-
	ant-I	Guo et al., 2017	-	-
Streptomycin	*aadA*	Guo et al., 2017	-	-
	ant6	Guo et al., 2017	-	-
	strA	Ouoba et al., 2007	-	-

3.2. Hemolysis

The wild type *L. paraplantarum* KCCM 11826P strains were cultured in a blood agar plate for the result of hemolysis activity. Compared to the three control strains that have the abilities of α-hemolysis, β-hemolysis, and γ-hemolysis, *L. paraplantarum* KCCM 11826P was confirmed to have no hemolysis activity (Fig. 2.1.).

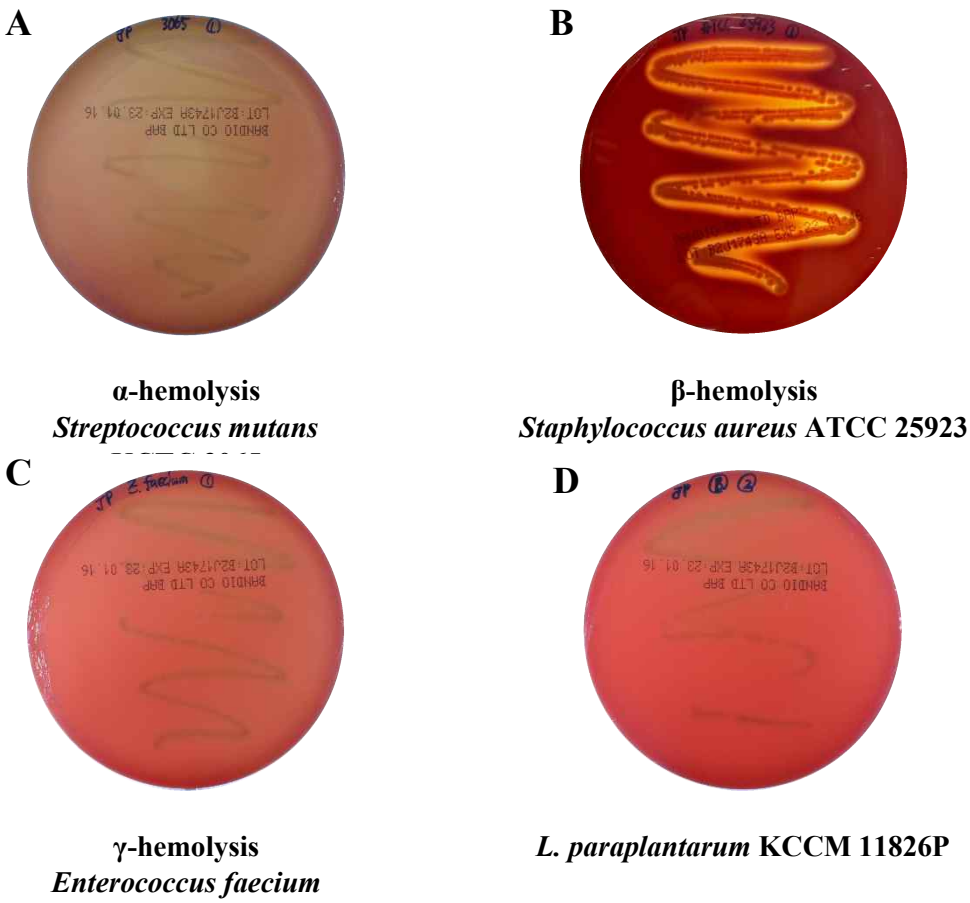


Fig. 2.1. Hemolysis activity (A) α-hemolysis activity of *S. mutans* KCTC 3065. (B) β-hemolysis of *S. aureus* ATCC 25923. (C) γ-hemolysis activity of *E. faecium*. (D) γ-hemolysis activity of *L. paraplantarum* KCCM 11826P.

3.3. Cell viability test

3.3.1. Acid tolerance test

By multiplying the quantity of CFU by the dilution factor, the number of viable bacteria was determined using CFU/ml (Table 5). At pH 4, viable bacteria ($log_{10}CFUml^{-1}$) recorded 101.85% of survival rate after incubation for 4 h. Similarly, at pH 3, the survival rate was 99.87%. Even under strong acid conditions such as pH 2, the *L. paraplantarum* KCCM 11826P showed a high survival rate of 97.2%. In previous studies conducted under the same conditions, *L. plantarum* OF101 showed a survival rate of 97.1%. *W. confusa* OF126 showed a survival rate of 91.2% (Adesulu-Dahunsi et al., 2018). As a result, we show that the *L. paraplantarum* KCCM 11826P is more acid resistant than other well-known lactic acid bacteria. And it can be seen that this strain survives well in a strongly acidic environment in the body.

3.3.2. Bile tolerance test

For bacteria to colonize and engage in metabolic activity in the host's small intestine, tolerance to bile salts is a requirement. It showed decent resistance to bile salt exposure for 4 h (Table 6). The survival rate of 90.79% was observed in 0.5% of bile salt. According to the prior studies, the *L. plantarum* MF 239 strain showed a survival rate of 86.00% in the presence of 0.3% of bile salt (Tokatlı et al., 2015). The *L. fermentum* LP3 strain showed a survival rate of 77.28% in the existence of 0.5% bile salt (Tulumoğlu et al., 2014). Therefore, the *L. paraplantarum* KCCM 11826P has better bile salt tolerance than the well-known probiotic strains.

Table 5. Survival rate of *L. paraplantarum* KCCM 11826P at various pH values determined by counts of viable bacteria over 2 hours interval Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of two repeated measurements from 2 independently replicated experiments, n=2.

Viable bacteria ($log_{10}CFUml^{-1}$)				**Survival rate (%)**		
pH	0 h	2 h	4 h	In this study	Adesulu-Dahunsi et al., 2018	
				L. paraplantarum KCCM 11826P	*L. plantarum* OF101	*W. confusa* OF126
2	8.31 ± 0.15	8.11 ± 0.15	8.08 ± 0.75	97.20	97.1	91.2
3	8.31 ± 0.80	8.30 ± 0.45	8.30 ± 0.00	99.87	-	-
4	8.30 ± 0.45	8.33 ± 0.15	8.45 ± 0.15	101.85	-	-

Table 6. Survival rate of *L. paraplantarum* KCCM 11826P at various bile salt concentrations as determined by counts of viable bacteria over 2 hours interval Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of two repeated measurements from 2 independently replicated experiments, n=2.

Viable bacteria ($log_{10}CFUml^{-1}$)				**Survival rate (%)**		
Bile salt concentration (%)	0 h	2 h	4 h	In this study	Tokatlı et al., 2015	Tulumoğlu et al., 2014
				L. paraplantarum KCCM 11826P	*L. plantarum* MF 239	*L. fermentum* LP3
0.1	8.36 ± 0.15	8.40 ± 0.89	8.57 ± 0.45	102.42	-	-
0.3	8.35 ± 1.15	8.24 ± 1.08	7.78 ± 1.00	93.19	86.00	-
0.5	8.35 ± 0.89	8.01 ± 0.80	7.58 ± 0.15	90.79	-	77.28

3.4. Metabolic properties

As a result of bioinformatics analysis using Geneious 6.0.6., it was confirmed that *L. paraplantarum* KCCM 11826P has the *larA* gene (DL-lactate racemase) (Desguin et al., 2014). And *bsh1* and *bsh3* gene (bile salt hydrolase) (Wang et al., 2021) were not existed (Table 7). BSH could break down bile salt, increasing the survival rate of bacteria in the body by inhibition of the bacterial membranes disruption. However, from a human perspective, excessive reduction in bile salt due to strong BSH activity makes fat digestion difficult.

D-lactate concentration of each probiotics strain were quantified. *L. paraplantarum* KCCM 11826P produced 16.634 g/L after culturing. *L. casei* ATCC 393 was the only strain which produced less D-lactate than *L. paraplantarum* KCCM 11826P. However, *L. plantarum* WCFS1 and KCTC 3108, well-known probiotics strains produced more D-lactate than *L. paraplantarum* KCCM 11826P (Table 8). Based on these results, the production of D-lactate does not make *L. paraplantarum* KCCM 11826P impossible to use as a probiotics. And many lactic acid bacteria produce D-lactate. *Lactobacillus* and *Leuconostoc* genera, which are well known as probiotics strains, could produce D-lactate (Vitetta et al., 2017). However, it may be risky for newborns, young children, and short bowel syndrome patients. So, the relevant wording should be specified in the ‘Precaution for ingestion’.

Table 7. The presence of the genes related to DL-lactate racemase and bile salt hydrolase

(–) means that there is no corresponding gene.

Product	Target Gene	Ref.	*L. paraplantarum* KCCM 11826P		
			Number	Start	End
DL-lactate racemase	*larA*	Desguin et al., 2014	1	1,641,180	1,642,454
Bile salt hydrolase	*bsh1*	Wang et al., 2021	-	-	
	bsh3		-	-	

Table 8. D-lactate production of probiotics strains Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of three repeated measurements from 3 independently replicated experiments, n=3.

Probiotic strain	D-lactate production (g/L)
***L. paraplantarum* KCCM 11826P**	16.63 ± 2.13
***L. plantarum* WCFS1**	17.11 ± 0.74
***L. plantarum* KCTC 3108**	24.06 ± 7.57
***L. casei* ATCC 393**	15.25 ± 3.97

3.5. Toxic metabolites

As a result of bioinformatics analysis using Geneious 6.0.6., it was confirmed that *L. paraplantarum* KCCM 11826P has no production of toxin (Table 9). Since *L. paraplantarum* KCCM 11826P does not exhibit physiological side effects in humans, so could be considered safe as a probiotics.

Table 9. The presence of the genes related to toxic metabolites

(–) means that there is no corresponding gene.

Product	Target Gene	Ref.	*L. paraplantarum* KCCM 11826P		
			Number	Start	End
Cereulide synthetase	*ces*	Ehling-Schulz et al., 2006	0	-	-
Non-hemolytic enterotoxin	*nhe*	Lindbäck et al., 2004	0	-	-
Hemolysin BL	*hbl*	L.S. Reis et al., 2013	0	-	-
Cytotoxin	*cytK1*	Koné et al., 2019	0	-	-
Histamine decarboxylase	*hdc*	M. Thomas et al., 2012	0	-	-
Tyramine decarboxylase	*tdc*	Moghadam et al., 2022	0	-	-

4. Conclusion

In chapter 2, *L. paraplantarum* KCCM 11826P's compliance with the probiotics guidelines as outlined by the FAO and WHO was verified. Through the MIC test, it was confirmed that *L. paraplantarum* KCCM 11826P is a safe probiotic strain that have no acquired antibiotic resistance genes. And compared to α- and β-hemolytic indicator bacteria, *L. paraplantarum* KCCM 11826P has no risk of destroying the host's red blood cells. Food typically goes from the stomach, which has a high acidity, into the intestine. The pH of the stomach changes to 2-3 when food enters. It takes about four hours to get through the stomach. Based on this, the degree of acid resistance of *L. paraplantarum* KCCM 11826P was tested. As a result, a high survival rate of 97.20% was recorded at pH 2. For a similar purpose, as a result of exposing the same time to various bile salt concentrations, it was found that the survival rate was 90.79% at 0.5% of the bile salt concentration. Each of these results means that *L. paraplantarum* KCCM 11826P could survive in harsh environments in the body better than other well-studied lactic acid bacteria. In addition, it was analyzed that there was no toxin component that could harm humans in the whole genome of *L. paraplantarum* KCCM 11826P. This strain only has one DL-lactate racemase related gene that other probiotics strains have also. When putting the above information together, *L. paraplantarum* KCCM 11826P passed the probiotics safety assessment.

Chapter 3.

Screening of the most efficient polyphosphate accumulating organisms using inverse metabolic engineering

1. Introduction

According to previous study, *L. paraplantarum* KCCM 11826P was found that could lower the serum phosphorus level (Moon et al., 2022). However, its polyphosphate accumulating mechanisms has yet to be clarified at the gene level. In chapter 2, inverse metabolic engineering was chosen to clarify potential possibilities. Fist, conventional mutagenesis was induced using nitro-N-nitrosoguanidine (NTG). Mutants were stained with 4',6-diamidino-2-phenylindole (DAPI) and then primarily screened for ideal mutants that could accumulate more polyphosphate in their cells with fluorescence activated cell sorting (FACS). After that, during the fermentation process in phosphorus rich medium, the ideal mutants were selected by quantifying the remaining phosphorus. Whole genome sequencing of the finally screened mutants revealed what kind of changes occurred in which position of the gene. Using 13-week-old male 5/6 nephrectomized Sprague-Dawley rats, it was confirmed that serum phosphate level was effectively reduced *in vivo* as well as *in vitro* compared to wild type strain.

2. Materials and Methods

2.1. Fermentation and growth conditions

L. paraplantarum SNUP7 was cultivated during preculture in De Man, Rogosa, and Sharpe (MRS, BD Difco, Franklin Lakes, NJ, USA) broth for 12 h at 30 ℃ in a shaking incubator (Vision, Bucheon, Korea) at 100 rpm. The inoculum was obtained from the preculture after centrifugation at 4 ℃, 4200 rpm for 20 m. After centrifugation, the pellet was twice cleaned with 1 x PBS buffer solution before being resuspended in MRS medium. The supernatant was then discarded. The main culture for the experiments was inoculated with a 1% (v/v) inoculum and cultured in the 100 ml MRS broth for 24 h at 30 ℃ in a shaking incubator at 100 rpm. The samples were collected every 4 h to measure growth. The degree of growth is analyzed by optical density ($O.D_{600}$) and colony-forming unit per milliliter (CFU/ml).

2.2. Mutagenesis

To perform mutagenesis, NTG was used. After culturing *L. paraplantarum* KCCM 11826P for 12 h, the supernatant was removed and the pellet was washed with 100 mM citrate buffer solution (pH 5.5). After then, washed pellet was treated with 100 mM citrate buffer which final concentration of NTG was 100 μg/ml for 30 min. After treatment, the treated pellet was obtained by centrifugation at 4 ℃, 4200 rpm for 20 m. The pellet was washed with 100 mM PBS buffer solution (pH 7.0). The resuspension was spread on MRS agar plate for 24 h at 30 ℃ after serial dilution. The potent mutant was obtained after incubation.

2.3. First screening using FACS

To screen the ideal mutants that have more useful polyphosphate accumulating ability, FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA) was used. The cells of the potent mutant were incubated in MRS broth overnight. After harvesting cell pellet, the cells were washed with cold PBS buffer solution and then resuspended in binding buffer solution. And the cells were stained with 2 mg/ml of DAPI staining solution for 30 min at room temperature. After incubation, pellet was washed twice with McIlvaine's buffer. The supernatant was discarded, and washed cell was resuspended with PBS buffer solution. This solution was immersed in the FACS tube. The cells were quantitated using a flow cytometry system with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. At least 10,000 events were recorded. The cells were expressed as a percentage of the total cell number (Fig. 3.1.).

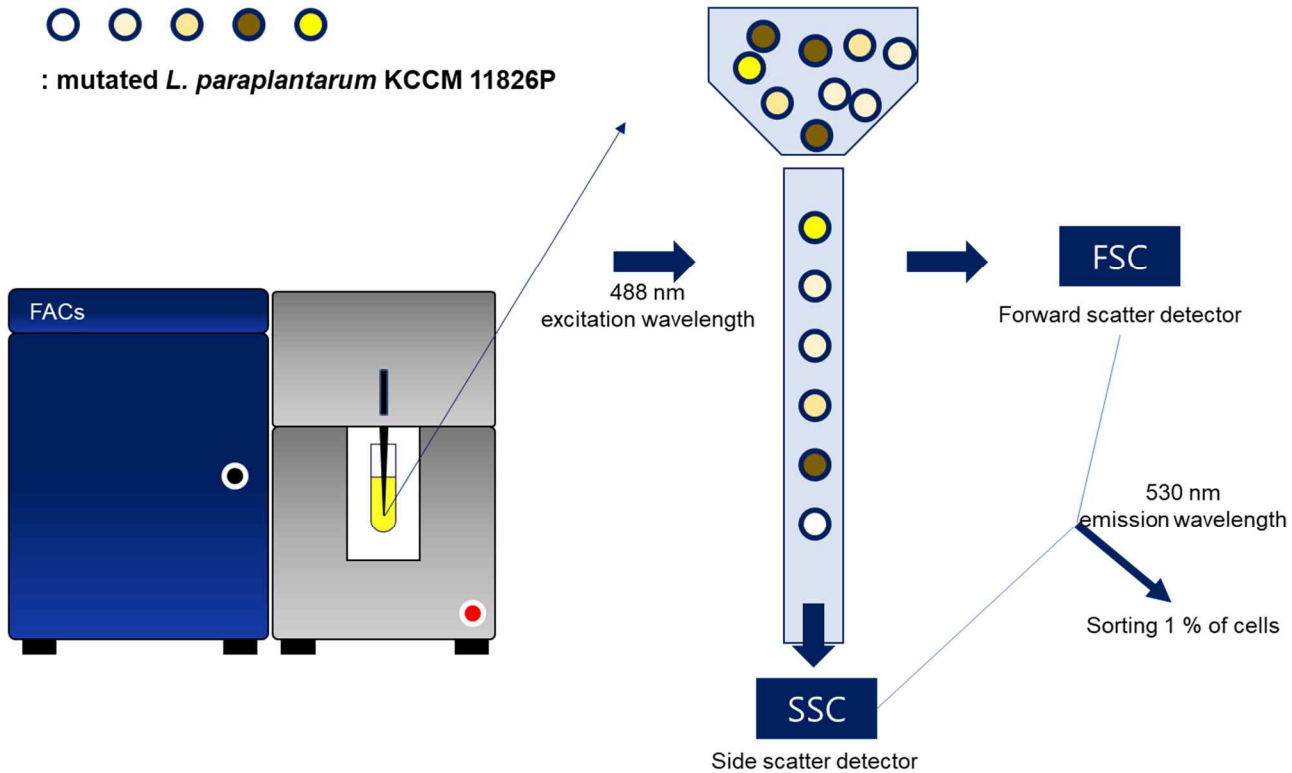


Fig. 3.1. Schematic diagram of FACS

2.4. Quantification remaining phosphorus in phosphate rich medium

The previous study showed that the higher amount of phosphate in the medium affected the capacity to absorb phosphate of *L. paraplantarum* KCCM 11826P proportionally (Moon et al., 2022). On this basis, the 300 colonies of mutant strain and *L. paraplantarum* KCCM 11826P were cultured during preculture in the MRS broth for 12 h at 30 ℃ in a shaking incubator at 100 rpm. The main culture was inoculated with a 1% (v/v) inoculum and cultured in the 5 ml MRS broth with potassium phosphate monobasic (PPM) concentrations of 4% (v/v). To estimate the level of phosphate consumption, Skalar San++ Auto analyzer with 1074 sampler (Skalar Analytical B.V., Tinstraat 12, Breda, The Netherlands) was used. After culturing, the of cell free supernatant (CFS) samples were collected and serial dilution was performed within the range of analysis. Then 10 ml of samples filtered with 0.45 μm syringe filter. All samples were analyzed as soon as possible, and when before analysis, they were kept within 24 h at 4 ℃. After device operation, all the sample pumps were washed using D.W for 30 m, then stabilizing process were performed with applicable reagents for 30 m. At the beginning of the analysis, the samples were mixed with a potassium peroxodisulfate and sodium hydroxide solution. Samples' organic phosphate was destroyed by UV light. The sample stream is mixed with sulfuric acid, and the mixture is then heated to 110 °C. Orthophosphate is created by the digestion of complex inorganic phosphates. A phospho-molybdic acid complex is created when diluted solutions of phosphate react with sodium molybdate, which was catalyzed by potassium antimonyl (iii) oxide tartrate, in an acidic medium. L(+)-ascorbic acid reduces this combination into a vividly blue-colored complex. At 880 nm, this compound was measured (Fig. 3.2.). The phosphate consumption level of the *L. paraplantarum* KCCM 11826P strain was used as a standard, mutant strains consumed more than this standard were selected first. The same analysis was conducted three times more to remove strains which had shown false-positive values from the selected strains. And the strains that continued to consume more phosphate than the standard were repeated five times for the same purpose.

2.4.1. Reagents and chemicals

Potassium peroxodisulfate, sodium hydroxide, boric acid, sulfuric acid (95-97%), ammonium heptamolybdate, potassium phosphate monobasic, potassium antimonyl (III) oxide tartrate and L(+)-ascorbic acid were all procured from Sigma-Aldrich. FFD6 was procured from Skalar. Oxidizing reagent was prepared by dissolving 6 g of sodium hydroxide in 900 ml of distilled water (D.W) first. After 25 g of potassium peroxodisulfate was totally dissolved, then 10 g of boric acid was added and filled up to 1 L with distilled water (D.W) and mixed. Sulfuric acid solution was prepared by diluting the 200 ml of sulfuric acid in 400 ml of D.W slowly. After cool down, it was filled up to 1 L with D.W, 1 ml of FFD6 was added and mixed. FFD6 solution was prepared by diluting 1 ml of FFD6 in 1 L of D.W and mixing. Sodium hydroxide solution was prepared by dissolving 140 g of sodium hydroxide in 800 ml of D.W. The solution was filled up to 1 L with D.W and mixed. Ammonium heptamolybdate solution was prepared by diluting 40 ml of the sulfuric acid in 800 ml of D.W. And then, ammonium heptamolybdate was added and dissolved. It was filled up to 1 L with D.W, then 2 ml of FFD6 was added and mixed. Potassium antimonyl (III) oxide tartrate stock solution was prepared by dissolving 300 mg of potassium antimonyl (III) oxide tartrate in 80 ml of D.W. Then it was filled up to 1 L with D.W and mixed. L(+)-ascorbic acid solution was prepared by dissolving 4.5 g of L(+)-ascorbic acid in 200 ml of D.W. 5 ml of potassium antimonyl (III) oxide tartrate stock solution was added and it was filled up to 250 ml with D.W and mixed. Phosphate stock solution was prepared by dissolving 439.4 mg of potassium phosphate monobasic in 800 ml of D.W. It was filled up to 1 L with D.W and mixed. This stock solution is 100 mg P/L. Working solutions were prepared by diluting phosphate stock solution. The concentrations of each working solution were 0.2, 0.5, 1.0, 2.0, and 5.0 mg P/L. All the reagents except working solution were used only for 1 week after preparation. Working solutions were prepared daily.

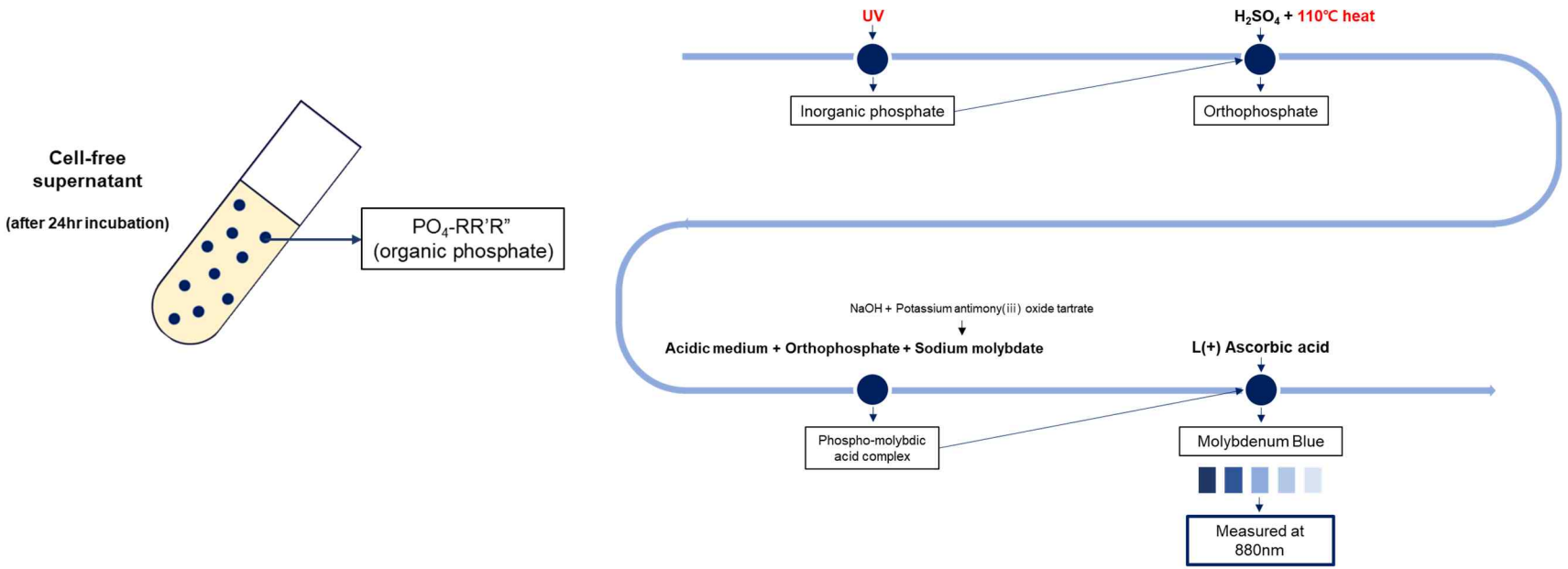


Fig. 3.2. Schematic diagram of ascorbic acid method

2.5. *In vitro* intracellular polyphosphate quantification using DAPI fluorescence

Moon et al. have lately shown that there was granular polyphosphate in the cells of *L. paraplantarum* KCCM 11826P. To determine intracellular polyphosphate concentration, the method of Kulakova was referenced with slight modification (Kulakova et al., 2011). The bacterial cells (0.5-1 x 10^9 CFU/ml) of selected mutants from second screening process were harvested at 10,000 rpm for 15 m at 4℃. After rinsing in 50 mM HEPES buffer solution (pH 7.5), the pellet was applied pretreatment that freezing cells at -20 ℃ and then thawing them at ambient temperature. Treated pellets were resuspended in DAPI assay buffer solution to stain intracellular polyphosphate. DAPI-polyphosphate fluorescence was measured using black 96 well plates in a Tristar^{2}S LB 942 multiplate reader (BERTHOLD tech, Bad Wildbad, Germany) supplied with excitation and emission filters of 420 nm and 535/25 nm respectively. Fluorescence intensity is stated as relative fluorescence units (r.f.u).

2.5.1. Reagents and chemicals

1 M HEPES (pH 7.5) and 1 M HEPES-KOH (pH 7.0) buffer solution were purchased from Biosesang (Biosesang, Sungnam, Korea). KCl was purchased from Daejung (Daejung Chemical and Metals Co. Ltd., Siheung, Korea). DAPI was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). HEPES and HEPES-KOH buffer solution was diluted to concentrations of 50 mM and 20 mM respectively using D.W. KCl solution was prepared using D.W followed by sterilization. DAPI assay buffer was prepared with 150 mM KCl, 20 mM HEPES-KOH (pH 7.0) and DAPI solution to a last concentration of 10 μm.

2.6. Animal test

2.6.1. Animals

To confirm whether the selected mutant strains absorb more phosphate than WT strain even in *in vivo* test, the method of Moon et al. was conducted with slight modifications (Moon et al., 2022). 13-week-old male 5/6 nephrectomized (Nx) and sham-operated Sprague-Dawley rats (Central Lab Animal inc., Seoul, Korea) were kept in a specific pathogen-free facility under a 12-h light-dark cycle. All the animal tests were evaluated and approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-220331-7-2). The Nx rats undertook a 2-step surgery. One-thirds of the upper and lower left kidney was amputated when the rats were 8 weeks old One week after, the previous stages of surgery were followed to perform a entire right kidney removal. At each stage of surgery, they were prescribed 5 mg/kg of enrofloxacin the day and 0.05 mg/kg of buprenorphine for 2 days after operation. The sham-operated group was subjected to a simple laparotomy. All operating processes were performed under inhaled 1.5-2.0 % of isoflurane anesthesia. All rats went through a period of stabilization for one week and fed high-protein (30 gm%) and high-phosphorus (1.2 gm%) diet. One week after stabilization, the rats were divided into 4 exploratory groups; Nx group that administered probiotics (Nx+P group), Nx group that administered the vehicle (Nx+V group), sham group that administered probiotics (S+P group), sham group that administered the vehicle (S+V group) (Fig. 3.3.), n=8 per group. The probiotics (SNUP7, $0.5x10^9$ per day) were microencapsulated and fed by oral gavage once a day in the morning for 6 weeks. The animals were provided to eat ad libitum and were given free access to water. Blood samples to test for BUN, creatinine, calcium, phosphorus, and indoxyl sulfate were taken at baseline and every 2 weeks. At the end of the test, all rats were sacrificed using carbon dioxide inhalation. Blood, urine, stool, and kidney samples were collected for analysis. Body weight, food, and water intake were recorded every week.

2.6.2. Biochemical analysis of animal samples

2.6.2.1. Kidney function

Blood for biochemical analysis was collected from the jugular vein without anesthesia and heart at 0, 2, 4, and 6 weeks. BUN, creatinine, calcium, and phosphorus in serum were measured using CDx analyzer (IDEXX Laboratories, Inc. Catalyst Dx Analyzer, Westbrook, AZ, USA). When the sample slide and serum of the rats were inserted into the analyzer, the CDx sprays the serum exactly onto the slide. And the analyzer measures the color level of the slide.

2.6.2.2. Kidney injury level

Left kidney were fixed in 10 % neutral buffered formalin (Bio-solution, Suwon, Korea) for 12 h. Tissue trimming was performed to finely cut some of the kidney to make tissue slides. In the stage of tissue processing, dehydration was carried out with ethanol to dissolve fat tissue in the kidney. And then xylene, chloroform, and benzene were used to increase the permeability of paraffin. After embedded in paraffin, H&E staining was performed to estimate renal pathological injury.

2.6.3. Probiotics and vehicle preparation

After culturing of *L. paraplantarum* SNUP7 for 18 h, cell pellet was obtained by centrifuging at 4000 rpm, 4 ℃ for 20 m and washed twice using 1X PBS buffer solution. Cell pellet was resuspended with 20% glucose solution for primary coating. Centrifugation with same conditions was performed, and supernatant was

discarded. 10 % skim milk solution was used as secondary coating and cryoprotectant. Resuspended samples were stored at -80 ℃ for 12-24 h. After then, the samples were freeze-dried in a freeze drier at -40 to -30 ℃, 0.2 mbar for 4 days. Freeze dried powders were stored at -80 ℃. Vehicle was skim milk solution. Probiotics solution was prepared by dissolving freeze dried powders in PBS buffer solution. Vehicle solution was prepared by diluting 10% skim milk solution with PBS buffer solution. Skim milk concentrations in probiotics and vehicle solution was same.

2.7. Error correction of *L. paraplantarum* KCCM 11826P and Resequencing of *L. paraplantarum* SNUP7

The genomic DNA of *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 were extracted by the Genomic DNA Purification Kit according to the manufacturer instructions. The whole genomic DNA was sequenced by next-generation sequencing (NGS) using the HiSeq X Ten platform (Illumina, 5200 Illumina Way, CA, USA) at Macrogen (Seoul, Korea). The genomic DNA of *L. paraplantarum* KCCM 11826P was for error correction with existing FASTA file from the previous study (Moon et al., 2022). Error correction was performed using Pilon (v1.21) software. After whole genome was assembled, the location of protein-coding sequences, tRNA genes, and rRNA genes were identified. Then their functions were annotated. After error correction was completed, the genomic DNA of *L. paraplantarum* SNUP7 was for whole genome resequencing with reference to calibrated *L. paraplantarum* KCCM 11826P's sequences. SnpEff (v4.3t) tool was used for annotating possible effects on genes that can be caused by variants identified through mapping. This software generated the following results; genes and transcripts affected by the variants, location of the variants, and affect to protein synthesis by the variants (e.g. generating a stop codon).

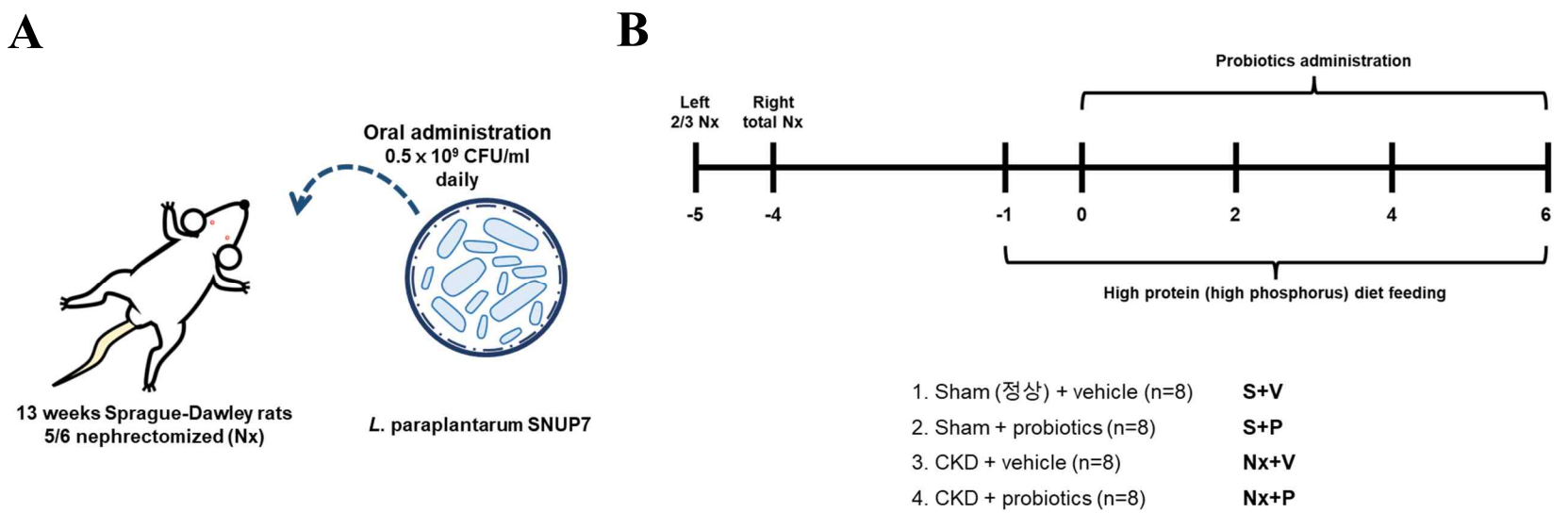


Fig. 3.3. Scheme for animal test (A) Animal model and probiotics strain. (B) Schedule and categorization of animal test.

3. Results

3.1. Fermentation and growth conditions

L. paraplantarum KCCM 11826P was cultivated with 100 ml of MRS broth at 30 ℃, 100 rpm for 24 h after preculture. Fig. 3.3. shows the growth results obtained under optimum growth conditions. The set strain of each $O.D_{600}$ were inoculated to MRS agar plate after serial dilution from 10^{-1} to 10^{-7}. The complete standard curve of CFU:$O.D_{600}$ is described in Fig. 3.4. The curve shows that $1x10^8$ CFU/ml of the strain was nearly similar as $O.D_{600}$ 0.1 and its R^2 is around 0.9829.

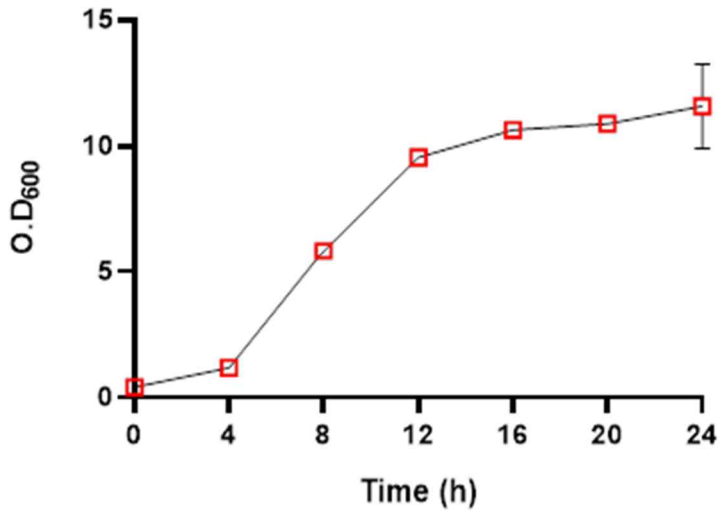


Fig. 3.4. Growth curve of *L. paraplantarum* KCCM 11826P at 30 ℃, 100 rpm for 24 h All values indicated the average of each fermentation and error bars depicted standard deviations.

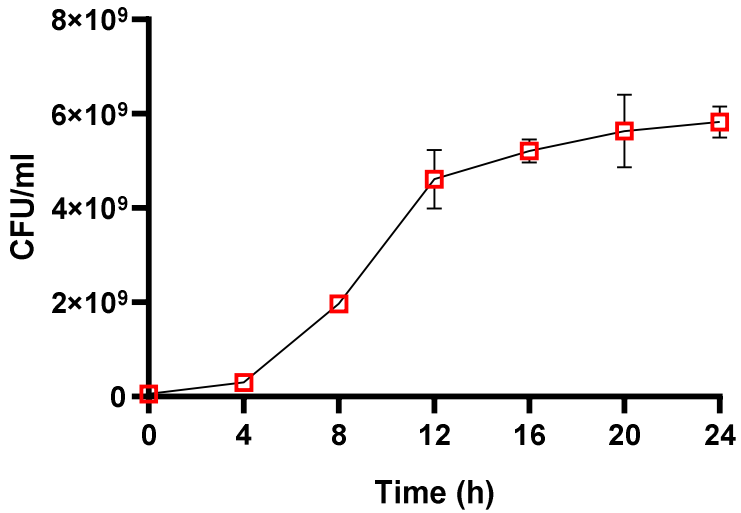


Fig. 3.5. Growth curve of *L. paraplantarum* KCCM 11826P at 30 ℃, 100 rpm for 24 h All values indicated the average of each fermentation and error bars depicted standard deviations.

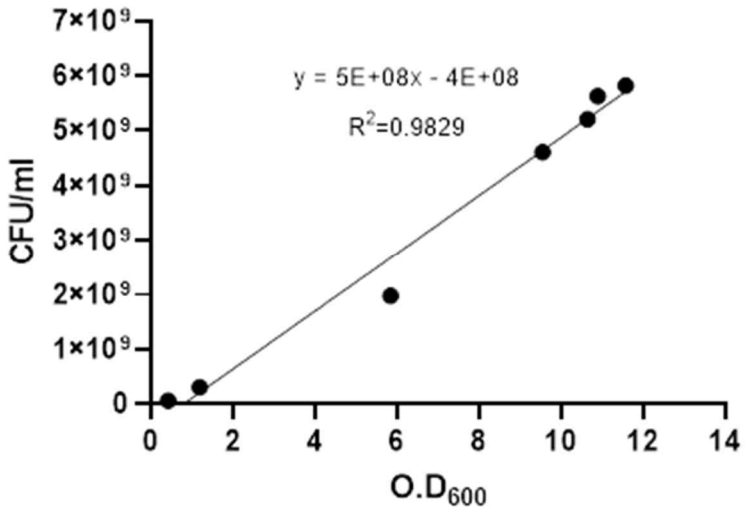


Fig. 3.6. CFU:O.D$_{600}$ correlation curve of *L. paraplantarum* KCCM 11826P

3.2. First screening using FACS

Intracellular polyphosphate of mutated *L. plantarum* KCCM 118265P was measured through FACS. According to the fluorescence intensity, mutated *L. plantarum* KCCM 118265P group representing the top 1% of values was separated. The selected mutant group was repeated this process. A total of 6 repeated experiments were performed, and abnormalities caused aggregation, making it impossible to proceed with the experiment. However, it was confirmed that the proportion of higher fluorescence intensity in the sixth staining group was higher than that of the first group (Fig. 3.7.).

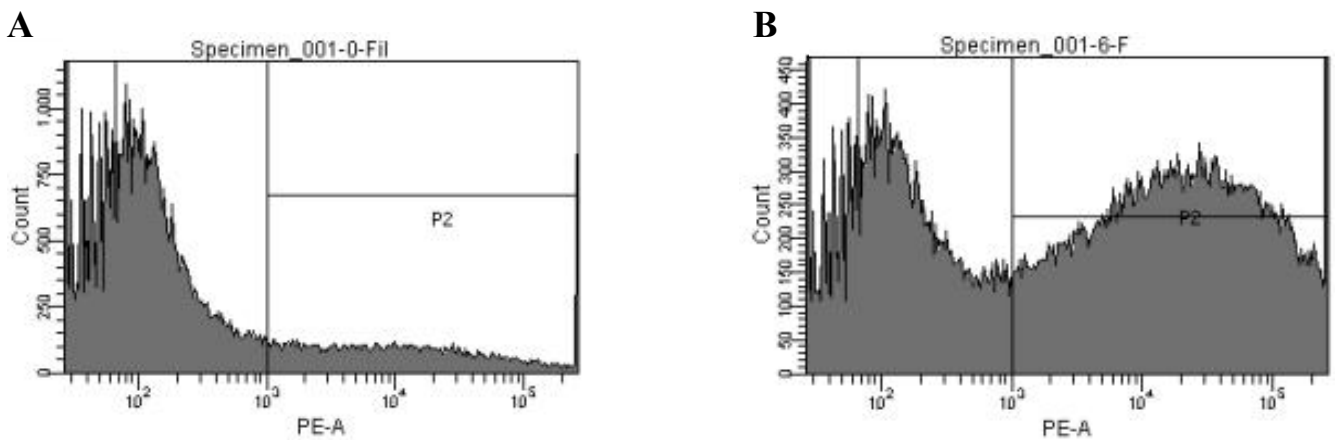


Fig. 3.7. FACS results (A) First stained group (B) Sixth stained group

3.3. Quantification remaining phosphorus in phosphate rich medium

Based on five *L. paraplantarum* KCCM 11826P strains, the phosphorus (P) absorption ability of 300 candidate mutant strains was compared. A single analysis without repetition showed that 195 out of 300 mutant strains absorbed more P in the phosphate rich medium than five *L. paraplantarum* KCCM 11826P strains (Fig. 3.8.). Among these, the 30 strains with the best P absorption ability were separated. These strains were independently cultured three times, and the same analysis was performed simultaneously to remove false negative values. After this step, 13 strains were screened again on the same basis. For the same reason as the previous step, they conducted five repetitive experiments. It was confirmed that the tendency to absorb more P than *L. paraplantarum* KCCM 11826P was consistent. Finally, the seven candidate mutant strains that absorb the most P in the screening step were selected. On average, they absorbed 1 to 2 g/L more P than *L. paraplantarum* KCCM 11826P (Fig. 3.9.).

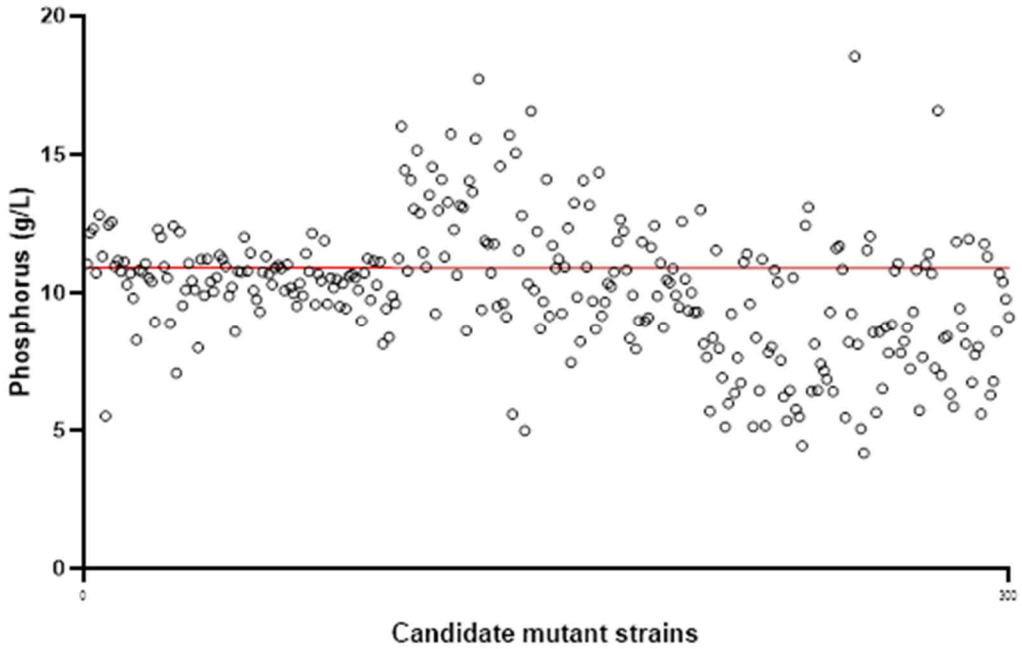


Fig. 3.8. Amount of residual P in the phosphate rich medium after culturing of 300 candidate mutant strains in the first step
(○) shows the amount of residual P in the supernatant of each mutant strain, (–) shows the average amount of residual P in the supernatant of *L. paraplantarum* KCCM 11826P.

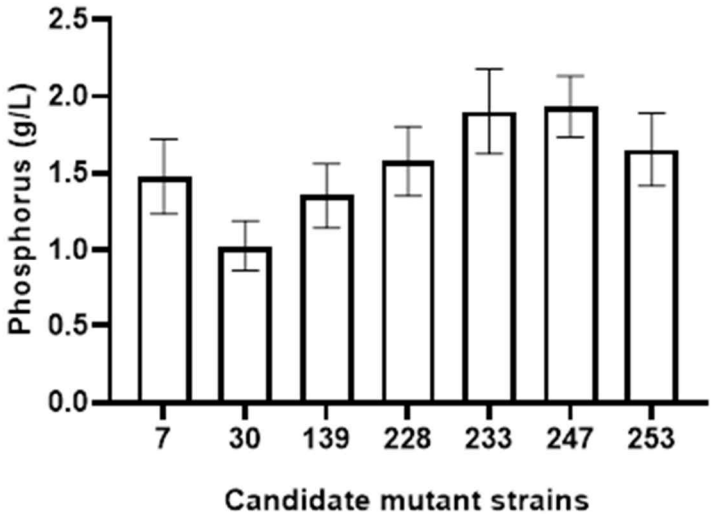


Fig. 3.9. The difference in the amount of P absorbed by *L. paraplantarum* KCCM 11826P and candidate mutant strains in the final analysis Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of five repeated measurements from 5 independently replicated experiments, n=5.

3.4. *In vitro* intracellular polyphosphate quantification using DAPI fluorescence

This experiment was conducted to determine whether the amount of extracellular P and intracellular polyP were correlated. The higher the measured fluorescence in tensity, the higher the polyP concentration in the cell. It was confirmed that the 7 mutant strains selected in the preceding experiments, exhibited higher fluorescence intensity than *L. paraplantarum* KCCM 11826P except strain 30 (Fig. 3.10.). Judging from this, it was observed that the selected 6 mutant strains absorb more surrounding P than *L. paraplantarum* KCCM 11826P owing to cross-validation.

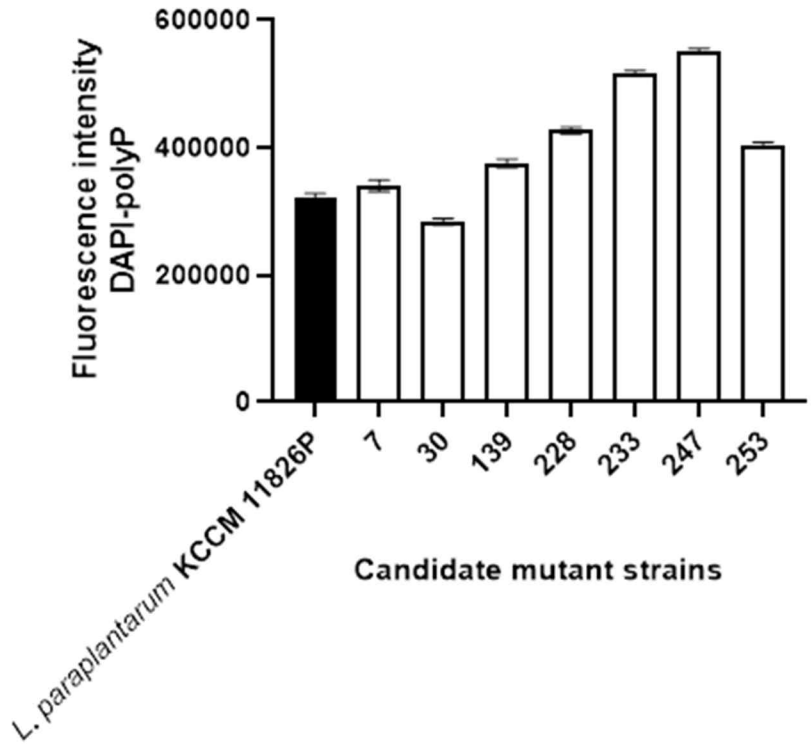


Fig. 3.10. The fluorescence intensity of *L. paraplantarum* KCCM 11826P and selected mutant strains Each value in the table represents the mean value ± Standard Deviation (SD). Each data point is the average of three repeated measurements from 3 independently replicated experiments, n=3.

3.5. Animal test

Moon et al verified that *L. paraplantarum* KCCM 11826P significantly reduce serum phosphorus level in CKD rats (Moon et al., 2022). Since *L. paraplantarum* SNUP7 absorbed more P in the medium than *L. paraplantarum* KCCM 11826P, *in vivo* study was performed whether it showed the same tendency.

Weight, water intake, and food intake were measured weekly in the health status category. It was found that the Nx group consumed twice as much water as the S group (Fig. 3.14b). The reason for this is believed to be that when drinking a large amount of water, the level of vasopressin hormones in the body decreases and the following mechanism that lowers blood pressure (Clark et al., 2015). However, the body weight and the food intake were not clearly different for each group. As a result, these mean that all groups maintained their health status during the experimental period (Fig. 3.14a. and c). In blood test, Nx groups had elevated levels of serum BUN and creatinine after a high-phosphate diet than the S groups (Fig. 3.15a. and b). Fig. 3.15c demonstrates that the level of serum phosphorus in the Nx rats increased drastically over time in comparison to that in the sham-operated rats. However, the increase was greatly slowed at six weeks in the Nx group that received *L. paraplantarum* SNUP7 (Nx + V 13.1 ± 2.41 vs. Nx + P 9.10 ± 1.05 mg/dL, $P < 0.001$). The serum calcium level was similar in all four groups (Fig. 3.15d.).

The kidney block from the Nx rats group with vehicle showed a visual difference compared to other groups. As a result of H&E staining to confirm the degree of pathological injury, the Nx+P group showed a degree similar to that of the sham-operated rats compared to the Nx+V group (Fig. 3.16.).

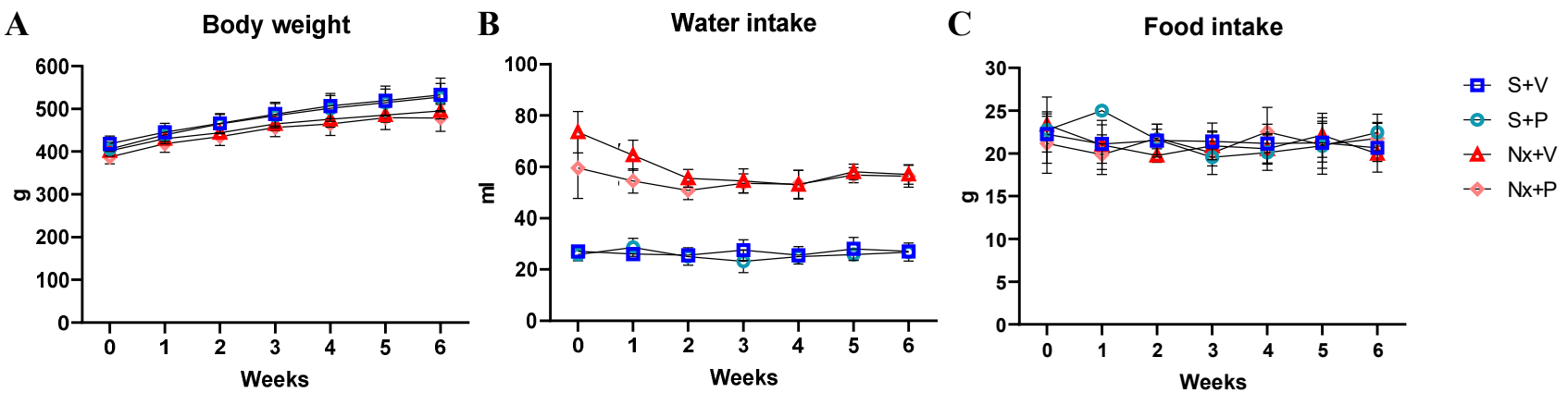


Fig. 3.11. The health condition of all groups (A) The body weights for 6 weeks. (B) The water intake for 6 weeks. (C) The food intake for 6 weeks. Values are expressed as means ± SEMs (*n*=8)

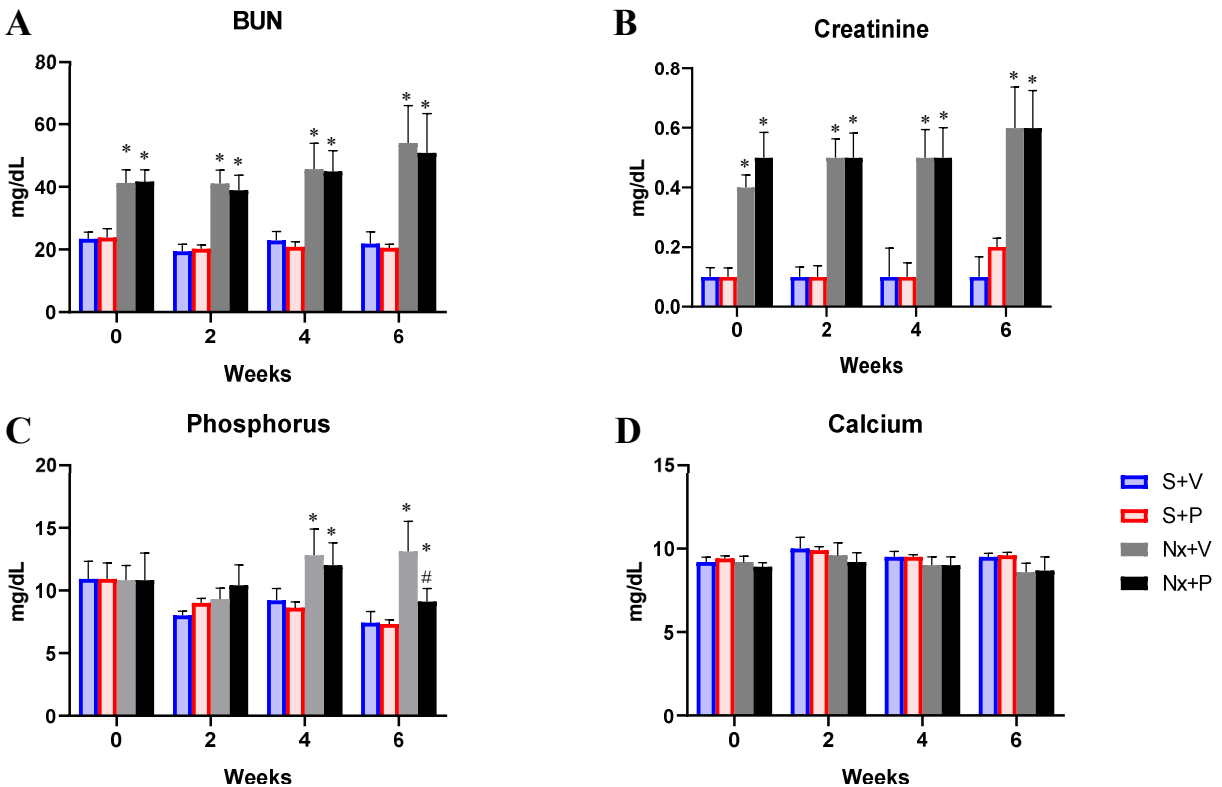


Fig. 3.12. Effect of probiotics administration in animal test (A) The serum BUN levels of the 4 groups: S+V (sham+vehicle), S+P (sham+probiotics), Nx+V (5/6 Nx+vehicle), Nx+P (5/6 Nx+probiotics) *$p < 0.001$ vs. S, S+P groups. (B) The serum creatinine levels. *$p < 0.001$ vs. S, S+P groups (C) The phosphorus levels. *$p < 0.001$ vs. S, S+P groups, #$p < 0.001$ vs. Nx+V group. (D) The calcium levels. Values are expressed as means ± SEMs (n=8)

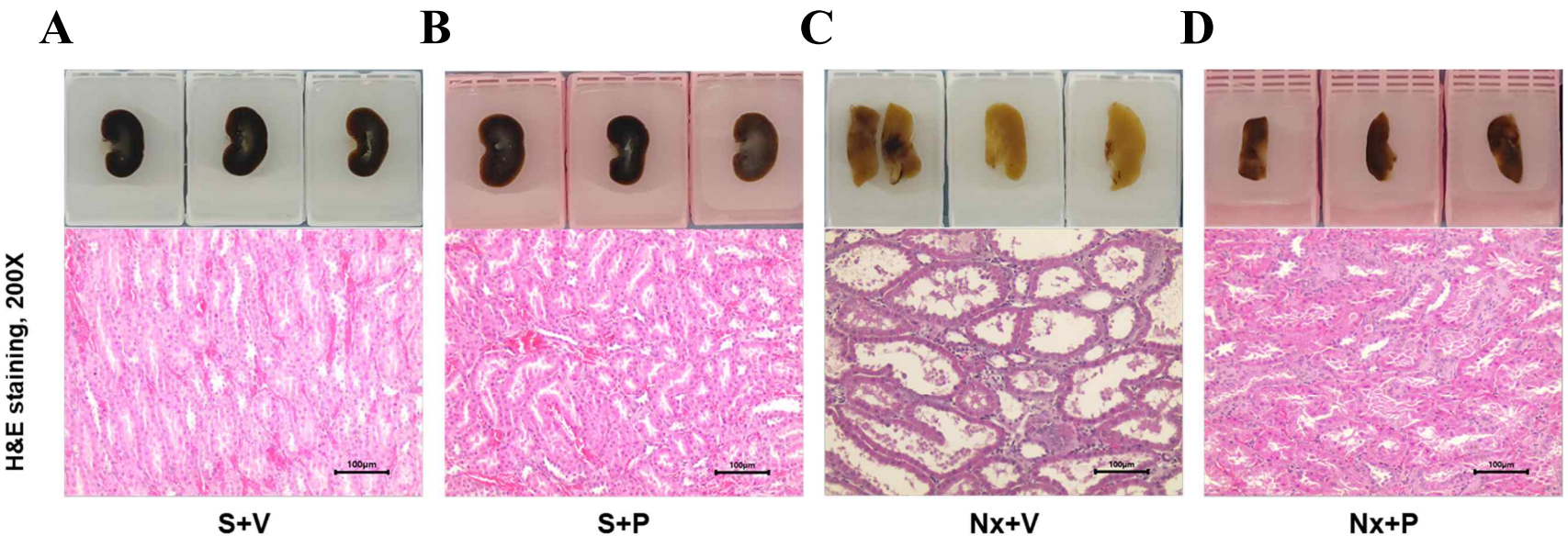


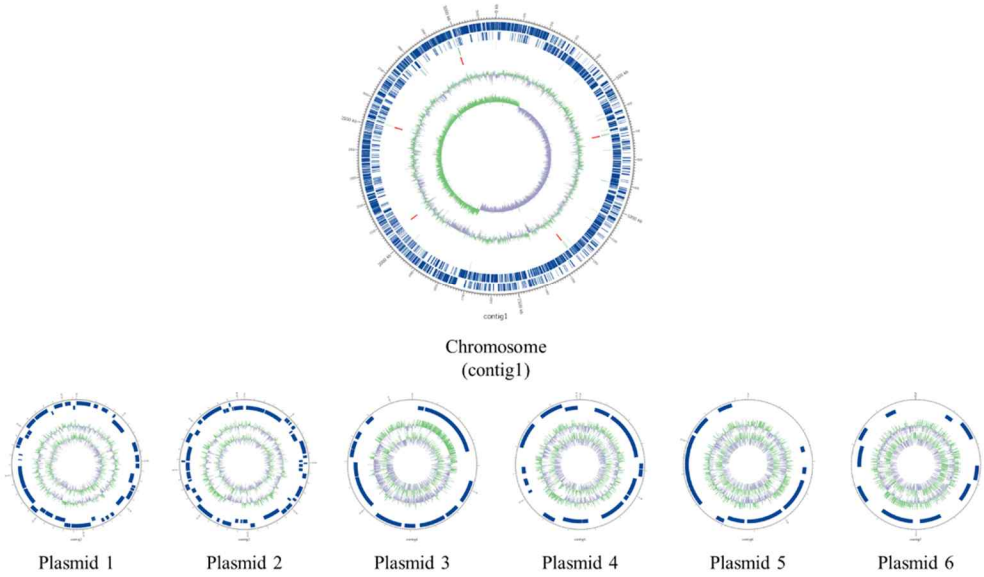
Fig. 3.13. Histopathological results of kidney (A) Representative kidney blocks and photomicrographs (200x) of H&E staining from S+V group. (B) Representative kidney blocks and photomicrographs (200x) of H&E staining from S+P group. (C) Representative kidney blocks and photomicrographs (200x) of H&E staining from Nx+V group. (D) Representative kidney blocks and photomicrographs (200x) of H&E staining from Nx+P group. Kidney samples from each group were randomly selected and photographed within the group.

3.6. Error correction of *L. paraplantarum* KCCM 11826P and Resequencing of *L. paraplantarum* SNUP7

After all screening processes were completed, strain 233 was selected from among the candidate mutant strains considered to have the best P absorption ability. This strain 233 was referred to as *L. paraplantarum* SNUP7. As shown in Fig. 3.11., the whole genome sequence of *L. paraplantarum* KCCM 11826P after error correction was generated using the HiSeq X Ten platform with Pilon (v1.21) software. The corrected whole genome of *L. paraplantarum* KCCM 11826P is composed of one 3,164,441 bp complete circular chromosome and six circular plasmids (plasmid 1 [41,383 bp], plasmid 2 [40,660], plasmid 3 [15,847], plasmid 4 [15,117], plasmid 5 [12,385], plasmid 6 [10,014]). GC content were 44.0 %, 40.1 %, 39.5 %, 40.6 %, 37.3 %, 36.0 %, 37.2 % respectively. Focusing on the chromosome and plasmids, there are 3,084 of CDS, 69 of tRNA, 16 of rRNA. These data were annotated by Prokka (v1.13), which is the pipeline that predict the location of protein expression genes, tRNA genes and rRNA genes and annotate its function.

The genome of *L. paraplantarum* SNUP7 was resequenced and compared to the genome of the reference strain *L. paraplantarum* KCCM 11826P. This is because to pinpoint the genetic variations that give the enhanced effect on P absorption of *L. paraplantarum* SNUP7. 2,718 single-nucleotide polymorphisms (SNPs) were found as a result of this comparison. The synonymous variations and mutations in unclear locations, which is considered to have little effect on protein activity, were eliminated. After trimming, the variations of genes related to protein folding and elongation of translation were found. In the sequence encoding chaperone protein DnaK (*dnaK*) and 60 kDa chaperonin (*groL*) related to protein folding, 14 and 8 bases, respectively, were altered due to the missense mutation (Table 10). Elongation factor Tu (*tuf,* EF-Tu), related to the elongation of the translation, was altered by missense mutation in 34 bases and nonsense mutation in 1 base (Table 11). According to Gray et al., polyP plays the role of chaperone, helping to fold proteins. In addition, it is shown that the longer the length of polyP, the more proportional the chaperone ability is (Gray et al., 2014). Caldas et al suggested that

EF-Tu plays a role in protein folding, which is the role of chaperones, as well as in the elongation of the protein translation (Caldas et al., 1998). Therefore, if the protein is not translated properly due to the malfunction of EF-Tu, an incomplete structure of the protein is produced (Fig. 3.12.). In this situation, if chaperone and chaperonin do not perform their role, the activity of proteins necessary for survival would be greatly reduced. To compensate for all this, *L. paraplantarum* SNUP7 would absorb more P than wild-type strain and accumulate polyP in the cell. Thus, the substance will be able to help the protein to be activated (Fig. 3.13.).



Contig Name	Bases (bp)	CDS	tRNA	rRNA	Alias
Contig1	3,164,441	2,932	69	16	Chromosome
Contig2	41,383	45	0	0	Plasmid 1
Contig3	40,660	52	0	0	Plasmid 2
Contig4	15,847	15	0	0	Plasmid 3
Contig5	15,117	18	0	0	Plasmid 4
Contig6	12,385	10	0	0	Plasmid 5
Contig7	10,014	12	0	0	Plasmid 6
Total	3,299,847	3,084	69	16	

Fig. 3.14. The circular genome graph of *L. paraplantarum* KCCM 11826P As the result, the whole genome of *L. paraplantarum* KCCM 11826P composed of seven contigs include one chromosome and six plasmids. From the inner ring to the outer ring, the first circle describes the GC skew (G+C/G-C), which means the replication starts. The second circle depicts GC contents, and the third, fourth circle indicate rRNA, tRNA. The fifth and sixth circle indicate reverse coding sequence (CDS) and forward coding sequence respectively.

Table 10. Mutations present in evolved *L. paraplantarum* SNUP7 and genetic analysis of its relevance for protein folding

Chromosome	Position	Reference sequence	Alteration	Gene	Product	Variation type
I	48427	A	C	*dnaK*	Chaperone protein DnaK	Missense
	48428	C	G			
	48429	A	G			
	48434	A	T			
	48456	C	G			
	48470	G	T			
	48515	C	G			
	48517	A	C			
	48524	A	T			
	48534	G	A			
	48810	A	G			
	49293	C	T			
	49440	C	T			
	49575	A	T			
	1146003	T	A	*groL*	60 kDa chaperonin	Missense
	1146004	T	G			
	1146008	T	C			
	1146707	A	T			
	1146774	A	T			
	1146788	A	C			
	1146872	A	T			
	1147157	T	A			

Table 11. Mutations present in evolved *L. paraplantarum* SNUP7 and genetic analysis of its relevance for protein translation

Chromosome	Position	Reference sequence	Alteration	Gene	Product	Variation type
I	3095532	T	G	*tuf*	Elongation factor Tu	Missense
	3095709	G	T			
	3095748	C	T			
	3095778	A	T			
	3095780	C	G			
	3095799	A	G			
	3095800	C	T			
	3095805	C	T			
	3095819	C	A			
	3095826	G	A			
	3095859	C	T			
	3096011	A	C			
	3096045	T	A			
	3096298	A	C			
	3096299	G	T			
	3096306	A	T			Stop
	3096307	A	T			Missense
	3096310	G	C			
	3096321	A	G			
	3096328	G	C			
	3096363	T	A			
	3096402	A	G			
	3096414	A	G			
	3096428	C	A			
	3096468	A	G			
	3096469	C	A			
	3096471	G	A			
	3096483	C	G			
	3096484	A	C			
	3096490	C	T			
	3096507	A	T			
	3096508	C	T			
	3096535	A	G			
	3096552	G	A			
	3096553	T	C			

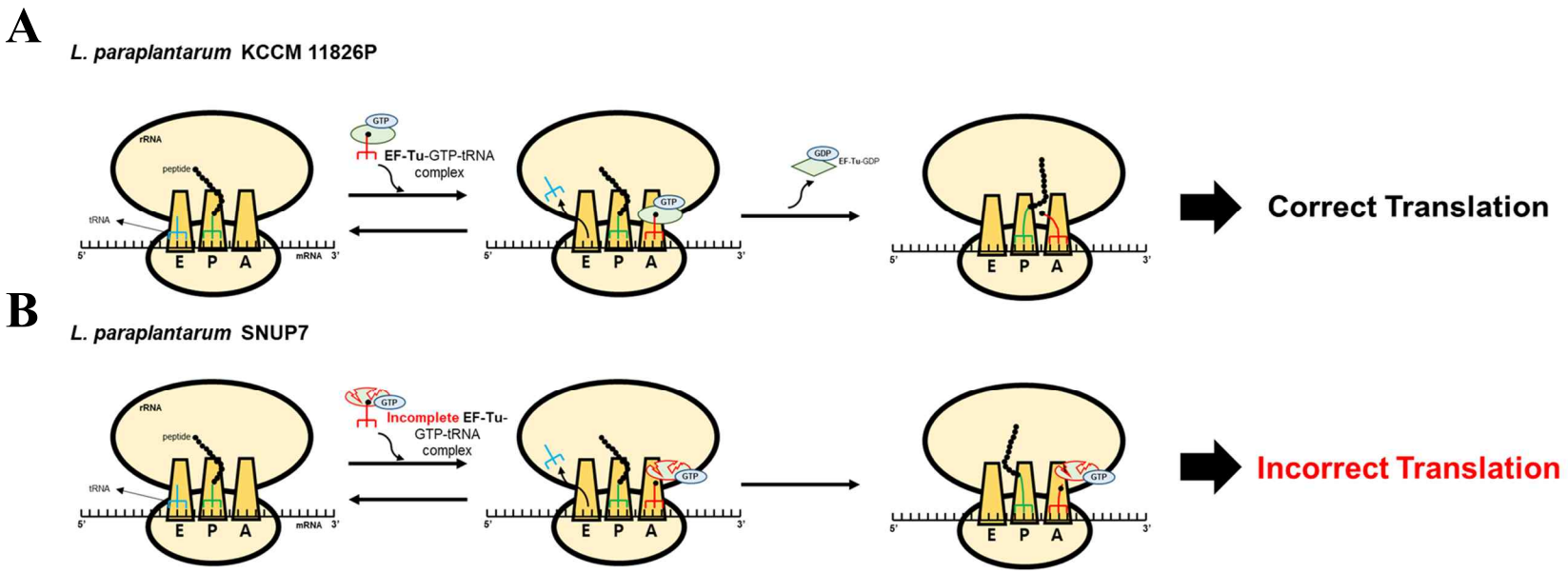


Fig. 3.15. Schematic diagram of protein translation in *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 (A) Protein translation in *L. paraplantarum* KCCM 11826P. (B) Protein translation in *L. paraplantarum* SNUP7.

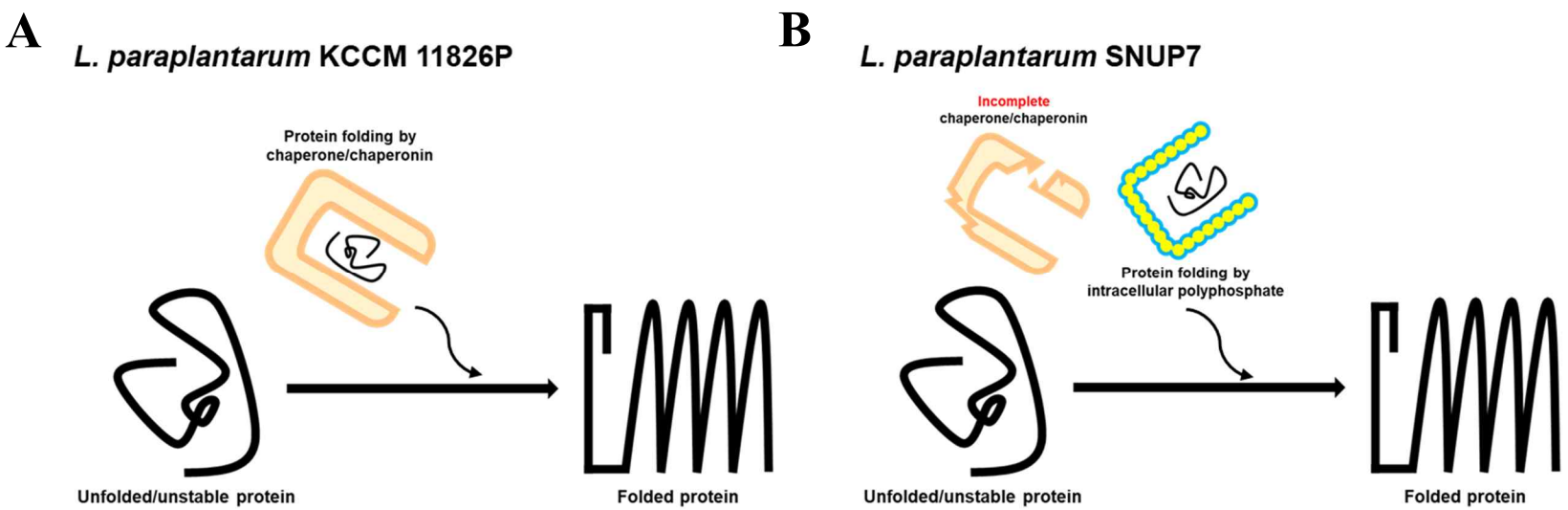


Fig. 3.16. Schematic diagram of protein folding in *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 (A) Protein folding in *L. paraplantarum* KCCM 11826P. (B) Protein folding in *L. paraplantarum* SNUP7.

4. Conclusion

In Chapter 3, genes associated with the ability to absorb the P of *L. paraplantarum* KCCM 11826P were identified. The optimal growth conditions of this strain validated in previous study were reconfirmed and applied to subsequent study.

Inverse metabolic engineering was used. In this process, it started with a random mutation using NTG. Among the mutants that occurred in this way, first, a group considered to absorb more phosphorus was selected using FACS. After that, the P absorption capacity of respective 300 colonies in the screened group were verified using automatic analyzer capable of processing large amounts at once. And the ability of the selected mutant strains was cross validated by staining of intracellular polyphosphate with DAPI. The final selected mutant, *L. paraplantarum* SNUP7, consumed 1.90 ± 0.28 g/L of P more than *L. paraplantarum* KCCM 11826P in phosphate-rich medium. And, *L. paraplantarum* SNUP7 was found to have mutations in the *dnaK*, *groL*, and *tuf* genes associated with protein folding and elongation of protein translation. As a result, this strain supplements the product of damaged genes with polyP produced by absorbing more P than the wild type.

L. paraplantarum SNUP7 was used in *in vivo* test to check whether it showed the same tendency not only in medium but also in the body. As a result of 6 weeks of oral administration, the serum P level in Nx+V group continued to increase rapidly, while the serum P level in Nx+P group tended to decrease (Nx + V 13.1 ± 2.41 vs. Nx + P 9.10 ± 1.05 mg/dL, $P < 0.001$). Histopathological results also showed that the kidney state of the Nx+P group seemed clearly similar to that of sham-operated group compared to Nx+V group.

5. Discussion

Existing treatments for hyperphosphatemia, the most fatal and frequently occurring complication in CKD patients, needs to be proposed an alternative due to the presence of side effects. The purpose of this study is to conduct safety test of *L. paraplantarum* KCCM 11826P and to secure mutant strain with increased absorption capacity as a result of improvement. Furthermore, genetic variations in mutant strains were identified. Safety evaluations was conducted according to the 'Probiotics guideline' with *L. paraplantarum* KCCM 11826P separated from Korean traditional fermented food in previous study. According to the safety evaluation procedure, first of all, it is necessary to determine the availability of strains based on the whole genome data. *L. paraplantarum* KCCM 11826P is an 'experienced' strain, so this strain could be used only if it meets the safety evaluation criteria except for toxicity test. However, unlike the experiments presented in the guidelines, toxic genes and BSH were evaluated only with the presence of genes, not phenotype verification. Phenotype verification experiments in guidelines are not mandatory and could be used if there is a scientifically equivalent test. After whole genome sequence analysis, it was confirmed that there were no related genes. As a result, this strain met all the criteria and does not have harmful effects to humans. Among the unexpected results, there was no antibiotic resistance to ampicillin, but strong resistance to aminoglycosides such as kanamycin and streptomycin in the MIC test. These characteristics were found to be intrinsic antibiotic resistance, as a result of using bioinformatical tools (Abriouel et al., 2015). Therefore, there is no possibility of transmitting antibiotic resistance to harmful bacteria around the intestine, so *L. paraplantarum* KCCM 11826P could be ingested safely. And *L. paraplantarum* KCCM 11826P has DL-lactate racemase, which is capable of producing D-lactate, but it was observed that activation level of this enzyme was similar or lower than the degree to which other probiotic strains produced D-lactate.

High-throughput screening was performed by comparing the difference in fluorescence intensity according to the interaction of DAPI-polyP and using the ascorbic acid method that could quantify residual P in the CFS. *In vitro* tests, *L.*

paraplantarum SNUP7, which absorbed 18.2% more P than *L. paraplantarum* KCCM 11826P was selected after screening processes. With regard to this result, it could be seen that *L. paraplantarum* SNUP7 has the ability to absorb more P from the surrounding environment than the *L. paraplantarum* KCCM 11826P at all times through independent additional iterative experiments. Compared to the results of previous studies, it could be absorb P significantly more than other beneficial lactic acid bacteria (Moon et al., 2022), but there is no standardized data on the comparison of probiotic strain for hyperphosphatemia such as strain L11 and B8, which were studied before (Anand & Aoyagi, 2019). Comparing the difference in phosphorus absorption between probiotic strains developed for similar purpose and commercial phosphate binders and *L. paraplantarum* SNUP7, it could further support the positive possibility of *L. paraplantarum* SNUP7 as the treatment.

In vivo test showed the effect of lowering the blood phosphorus level of Nx+P group to 30.5% compared to the Nx+V group without additional toxicities. This figure is significantly higher than that of the CKD rats group administered with *L. paraplantarum* KCCM 11826P in the same experimental method in previous study (Moon et al., 2022), which reduced the blood phosphorus level by 22% compared to the Nx+V group. For more accurate verification, a process of performing *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 administration at once and then comparing the decrease in blood phosphorus level should be required. And, probiotics research is being actively conducted not only on chronic kidney diseases but also on various diseases that threaten human health (Varsha et al., 2021). To support these studies, animal tests were also carried out, supporting the argument on a preclinical basis. However, as attempted in this study, administration of freeze-dried and powdered probiotics was rarely performed. This study would be a cornerstone of significant development in future research on probiotics for alleviating chronic diseases.

dnaK, *groL*, and *tuf* genes in *L. paraplantarum* SNUP7 were mutated. The malfunction of these genes affects the polyphosphate accumulation mechanism to compensate for incorrect protein production. But it is necessary to determine how the change in these genes are related to PPK, PPX, Pst system, PhoR, and PhoU that affect polyphosphate accumulation ability.

To supplement this study, several future works are needed. In chapter 2, it was demonstrated that there were no genes involved in toxin production. However, lactate dehydrogenase (LDH) assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) reduction assay are needed to determine whether toxicity-related phenotypes appear in Caco-2 or HT-29 cell. In the case of LDH assay, the degree of apoptosis of cells is measured by measuring the amount of LDH that comes out after cells are destroyed by toxins. MTT assay is a measure of cell apoptosis based on the principle that yellow colored MTT enters the living cell and is changed into purple formazan by mitochondrial reductase (Chen et al., 2017). And BSH also does not have related genes, but experiments need to be conducted to represent phenotypes. In 0.5% of bile acid-containing MRS agar plate, *L. paraplantarum* KCCM 11826P would be streaked and cultured. After that, if an opaque white colony is formed around the colony, BSH activity is considered. If positive, the secondary metabolite of deconjugated bile salt may be toxic, so care should be taken not to consume too much (Shehata et al., 2016).

In chapter 3, the fluorescence intensity levels indicating the degree of polyphosphate accumulation in cells per colonies were proven. However, in order to perform more accurate quantitative verification, it is essential to break the cell and check the degree of polyphosphate accumulation in *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7. In previous studies, it was confirmed that the concentration of indoxyl sulfate (IS) in the group which administered *L. paraplantarum* KCCM 11826P decreased (Moon et al., 2022). We should make sure that *L. paraplantarum* SNUP7 also has the same phenotype. IS is correlated with the indole produced by tryptophanase of intestinal microorganisms (Zhang & Davies, 2016). *L. paraplantarum* SNUP7 does not have tryptophanase, but it is required to determine the mechanism for what causes IS to decrease. If the above process is performed, clues to inhibition of kidney damage may be derived. In addition, understanding the mechanism that controls hyperphosphatemia following administration of *L. paraplantarum* SNUP7 might benefit from knowledge of the variations in FGF 23 levels (Stubbs et al., 2007). Fecal analysis should be conducted to confirm whether *L. paraplantarum* SNUP7 changes the microbial composition in the intestine in a direction beneficial to the host. The substance assay to determine if

there is actually phosphorus in this feces should be conducted to understand the process of treating hyperphosphatemia in *L. paraplantarum* SNUP7 in molecular biology. If the phosphorus concentration is detected higher in the feces of the probiotic administration group than in the control group, it could be assumed that *L. paraplantarum* SNUP7 has escaped to the feces with phosphorus.

Taken together, *L. paraplantarum* KCCM 11826P and *L. paraplantarum* SNUP7 are considered safe to be used as probiotics. And *L. paraplantarum* SNUP7 was observed to have improved P absorption capacity than *L. paraplantarum* KCCM 11826P. So that these probiotic PAOs showed potential for treatment as a bio-phosphate binder in CKD patients.

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Abstract in Korean

만성신장질환 (CKD) 환자 사망 원인의 47%는 고인산혈증에 의한 심혈관질환이다. 고인산혈증을 치료하기 위한 기존의 치료 요법으로는 투석, 단백질 제한 식이요법, 그리고 인산염 결합제 등이 있다. 다만 이들은 CKD 환자들의 삶의 질을 떨어뜨릴 우려가 있고 부작용도 여러 차례 보고된 바 있다. 다중인산염 축적능을 지닌 프로바이오틱스는 앞서 설명한 단점을 상쇄시킬 수 있는 인산염 결합제의 대안으로 제시된다. *L. paraplantarum* KCCM 11826P 균주는 선행 연구를 통해 해당 균주가 우수한 다중인산염 축적능을 갖고 있는 것이 증명되었다. 이 균주는 식품의약품안전처의 '프로바이오틱스 기능성 원료 평가 가이드'에 의거하여 안전성 평가를 진행한 결과, 프로바이오틱스로써 사용되기에 안전하다는 것이 확인됐다. 또한, 인 흡수 기전을 파악하기 위하여 inverse metabolic engineering 을 적용한 고성능 스크리닝 실험을 통해 *L. paraplantarum* SNUP7 돌연변이 균주가 선별되었다. Resequencing 을 진행한 결과, SNUP7 균주는 *tuf*, *dnaK*, *groL* 이 변이되어 *L. paraplantarum* KCCM 11826P 보다 인 흡수가 1.90 ± 0.28 g/L 더 소비한 것이 관찰됐다. 동물 실험에서도, SNUP7 프로바이오틱스를 투여한 CKD 랫드 그룹의 (Nx+P) 혈중 인 농도가 위약을 투여한 CKD 랫드 그룹 (Nx+V) 보다 30.5 % 감소했다. 또한 Nx+P 그룹의 신장 손상이 저해되었으나, Nx+V 그룹의 신장 손상은 그러지 못했다. 따라서, *L. paraplantarum* KCCM 11826P 와 *L. paraplantarum* SNUP7 균주는 생물학적 인산염 결합제 역할을 수행할 수 있으며, CKD 증상 완화 및 환자의 삶의 질 향상에 기여할 가능성이 있다.