Master's Thesis of Science in Agriculture

Antimicrobial Effect of *Lactococcus lactis* subsp. *lactis* LDTM 6804 on Psychrotrophic Spoilage and Pathogenic Bacteria in Dairy Products

유제품 내에서 유산균 ***Lactococcus lactis*** **subsp. *lactis* LDTM6804**의 내냉성 부패 및 병원성 미생물 억제 효과

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Antimicrobial Effect of *Lactococcus lactis* subsp. *lactis* LDTM 6804 on Psychrotrophic Spoilage and Pathogenic Bacteria in Dairy Products

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Abstract

In dairy industry, microbiological spoilage related to the quality and safety of milk and milk products is still an important issue for consumers. The growing demand for safer dairy products has led to consider the use of biopreservatives, mainly lactic acid bacteria (LAB). LAB exert inhibitory effects on the growth of undesirable microorganisms or pathogenic bacteria producing a variety of antimirobial compounds including organic acids, hydrogen peroxide and bacteriocins. Therefore, the purpose of this study was to evaluate a Lactococcus strain isolated from raw milk as a potential bioprotective culture using the dairy product models.

Lactococcus lactis subsp. *lactis* LDTM6804 (*L. lactis* LDTM6804), due to the highest antimicrobial activities against psychrotrophic spoilage and pathogenic bacteria, was selected among 306 isolates from raw milk. Furthermore, *L. lactis* LDTM6804 was characterized and its whole genome sequencing was performed. Gene cluster involved in nisin Z biosynthesis was identified in chromosome of *L. lactis* LDTM6804. Protective effects of *L. lactis* LDTM6804 was examined with 10 % skim milk and cheese model. When *L. lactis* LDTM6804 (6 log CFU/ml) was added to skim milk, viable cell numbers in *Pseudomonas fluorescens*, *Burkholderia cepacia* and *Listeria monocytogenes*

were decreased by 9.29, 8.84, 4.93 log units, respectively after 14 d of storage at 15 ℃ compared to skim milk with reference strain *L. lactis* IL1403. Cheeses were manufactured and infected by *L. monocytogenes, Staphylococcus aureus and Escherichia coli,* respectively at approximately 5 log CFU/g. In addition, *L. lactis* LDTM6804 at 1 % was inoculated to cheese as an adjunct to the starter culture. After 25 d of storage, *L. lactis* LDTM6804 reduced the viable cell numbers of *L.monocytogenes*, *S. aureus* and *E. coli* by 0.42, 0.57 and 0.74 log units, respectively compared to control cheese without *L. lactis* LDTM6804. During fermentation of 10 % skim milk and ripening process of cheese, nisin activity throughout storage was assessed using quantitative real time PCR (qRT-PCR). An electronic nose apparatus was employed to investigate changes of flavour compounds by *L. lactis* LDTM6804. Our results demonstrate that *L. lactis* LDTM6804 exerts strong protective effects against psychrotrophic spoilage and pathogenic bacteria without organoleptic quality changes in dairy products. Further investigations on the optimum level of addition of the protective culture and inhibitory effect of fermentates or purified nisin of *L.lactis* LDTM6804 were required.

Keyword : Protective culture, Biopreservation, Nisin, Dairy product, Lactic acid bacteria, *Lactococcus lactis*
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Chapter 1. Introduction

The microbiota of raw milk is complex. Many microorganisms contribute to flavour, texture or health promoting perspective, while some lethal pathogenic bacteria are significant risks associated with the consumption of raw milk and raw milk-derived products. Also, some bacteria introduced from surrounding environment are able to grow at refrigeration temperature (7 ℃), called psychrotrophic bacteria and synthesize heat-resistant proteases and lipases influencing negatively on milk and dairy products quality, reduced yield of curd, undesirable flavour and texture (Samaržija et al., 2012). To eliminate disease causing microorganisms and psychrotrophic spoilage bacteria, pasteurisation or other treatments are performed. After these treatments, re-introduction in the form of starter or adjunct cultures is served to compensate deficiency of lactic acid bacteria (LAB) or other bacteria considered beneficial during fermentations (Quigley et al., 2013). Likewise, biopreservation by adoption of LAB controls the undesirable spoilage and pathogenic microorganisms and enhances the quality and safety of dairy products (Holzapfel et al., 1995). LAB exhibit a wide range of antimicrobial activities through production of antimicrobial substances (organic acids, hydrogen peroxide,

bacteriocins, enzymes, low-molecular metabolites), competition for essential nutrients and unknown effects (e.g. quorum sensing).

Lactococcus lactis strains are commonly used for starter cultures in cheese, fermented milk products, ripened cream butter and other dairy products. In addition to rapid acid forming ability, antimicrobial substances production is important feature of *Lactococcus lactis* to prevent spoilage and pathogenic bacteria. Large studies were carried out on various bacteriocins, which are the one of very active antimicrobial substances of *L. lactis* including nisin, lactococcin, lacticin and others. Nisin is already acknowledged as Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA) and used in dairy process (Moreno et al., 2000). Also, various researches on effectiveness and control activity against pathogenic bacteria of bacteriocin-producing *Lactococcus lactis* subsp. *lactis* were performed in cheese model (McAuliffe et al., 1999, Coelho et al., 2014, Furtado et al., 2015, Callon et al., 2016).

In this study, we screened the *Lactococcus lactis* subsp. *lactis* LDTM 6804 from raw milk, shown a broad and high antimicrobial activity and investigated the potiental as protective culture. Additionally, we validated the antimicrobial ability in skim milk and cheese model. Also genes associated antimicrobial activity were identified through the whole genome sequencing.

Chapter 2. Review of Literature

2.1. Microorganisms in dairy products

Due to its nutritional properties, milk serves as optimal medium for many microorganisms. Lactic acid bacteria (LAB) which ferment lactose to lactate, and psychrotrophic bacteria, a group of bacteria that proliferate during cold storage are dominant populations in raw milk. The most common LAB genera consist of *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus*. *Pseudomonas*, *Aeromonas* spp. and *Acinetobacter* spp. are accepted as dominant genera of psychrotrophic bacteria. Subsequent quality of dairy products is directly affected by microbiological composition of the raw milk. Specific microorganism can bring about the fermentation and affect variously on the sensory, texture, flavor and organoleptic properties of products. Some of them affect negatively on quality and shelf life. Psychrotrophic bacteria can grow under refrigeration temperature and secrete proteases and lipases resulting in spoilage. Also contamination with pathogens can lead to severe illnesses in human.

Milk microorganisms are transferred from various sources including the teat, milking equipment, bedding, feed and other environments. As a consequence, raw milk contains a diverse bacterial population (Quigley et al., 2013).

During milk fermentation, some microorganisms in milk are competitive and reach a dynamic equilibrium. Several core genera– *Lactobacillus*, *Streptococcus*, and *Lactococcus*– are predominant. *Lactobacillus* are basic LAB during milk fermentation which degrade the lactose, the major carbohydrate of milk. Additionally, other metabolic properties of the *Lactobacillus*, such as proteolytic and lipolytic capacities, contribute to the flavor and texture of fermented milk. *Streptococcus* are also one of the dominant genera in fermented milk, *Streptococcus thermophilus* is commonly used starter cultures in milk fermentation. *Lactococcus* account for high proportion especially in cheese. The major role of these LAB are the production of lactic acid, hydrolysis of casein, lipolysis of fat, and fermentation of citric acid (Zhong et al., 2016).

2.1.1. Lactic Acid Bacteria (LAB) in dairy products

The LAB in dairy products are classified by optimum growth temperature into two groups. Mesophilic LAB can flourish under 20–30 ℃ and thermophilic LAB have optimal growth at 30–45 ℃. LAB are used as starter cultures for rapid acidification in dairy products. Use of LAB contributes to microbiological safety and advanced organoleptic and structural properties. Lactococci are the prominent group of mesophilic LAB in manufacture of various cheese. Majority of cheeses starter

cultures based on single species, *Lactococcus lactis*. LAB can contribute to flavor of cheese through proteolysis and other biochemical process (Wouters et al., 2002).

2.1.2. Psychrotrophic spoilage and pathogenic organisms in dairy products

Milk is typically stored at refrigerating temperatures (around 4 ℃) to inhibit the growth of most bacteria except psychrotrophic bacteria. Psychrotrophic bacteria can flourish under these conditions and mainly causes the spoilage of milk. Extracellular proteases and lipases are produced by psychrotrophic bacteria leading to grey colour, bitter off-flavour, rancidity. *Pseudomonas* spp., is the most common genus of psychrotrophic bacteria and cause of milk spoilage. The frequently detected species of *Pseudomonas* are *Pseudomonas fluorescens*, *Pseudomonas gessardii*, *Pseudomonas fragi* and *Pseudomonas lundensis*. These bacteria constitute up to 70-90 % of the microbial population under refrigerating storage.

Also spore-forming milk contaminants consist of *Bacillus cereus*, *B. sporothermodurans* and *Geobacillus stearothermophilus*. *B. cereus* is a very common spoilage organism of milk products stored at refrigeration, causing off-flavour and curdling.

Pathogens can originate from the mammary gland or

associated lymph nodes of cows suffering from systemic diseases or infections and equipments. Intake of these microorganisms can cause of illnesses of varying severity. Common symptoms are fever, nausea, vomiting, diarrhea and abdominal pains. The main milkborne pathogenic bacteria which cause illnesses are *Salmonella* spp., *Listeria* spp., *E.coli*, *Campylobacter* spp., *Brucella* spp. or *Shigella* spp. Typically many pathogenic bacteria are removed after pasteurization, but remains the heat-stable bacteria and toxins. *Staphylococcus aureus* introduced to milk from the environment leads to illness through the heat-resistant enterotoxins which can endure pasteurization.

Psychrotrophic bacteria and pathogenic bacteria are related to hygiene of farm, including cow health, equipment cleanliness, overall farm and personnel sanitation, correct storage and subsequent processing of milk. The practices that prevent or limit the presence of them are crucial(Quigley et al., 2013).

2.1.3. Impact of psychrotrophic spoilage and pathogenic bacteria on quality and safety of dairy products

Refrigeration extension of raw milk (2-6 ℃) has considerable impact on its composition of microbial communities. Psychrotrophic bacteria can grow up to 10^7 CFU/ml even an initially low bacteria counts (<50,000 CFU/ml). They synthesize

thermostable extracellular and/or intracellular hydrolytic enzymes, regarded as common contamination of heat treated milk and dairy products after pasteurization. Spoilage of raw milk is the consequence of the undesirable of flavor, coagulation of milk proteins, and the accumulation of free fatty and amino acids. Undesirable texture, organic compounds production, reduced yields and limiting shelf life of dairy products are also the quality defect resulted from psychrotrophic bacteria. In addition, lipases such as lecithinase and other phospholipases of psychrotrophic bacteria disperse the fat globules membrane structure leading to emulsion degradation in milk. Although psychrotrophic bacteria are mostly regarded not to be pathogenic, certain groups which can produce toxins and/or are resistant to antibiotics are considered as opportunistic pathogenic bacteria (Samaržija et al., 2012).

One of the most common food-borne pathogenic bacteria, *Listeria monocytogenes*, causes listeriosis threatening unborn, newborn and immunocompromised individuals. Contamination with *L. monocytogenes* may pose a serious threat in foods such as freshly fermented dairy products(McAuliffe et al., 1999).

2.2. *Lactococcus lactis* subsp. *lactis*

2.2.1. *Lactococcus lactis* subsp. *lactis*

The genus *Lactococcus* belongs to the family *Streptococcaceae* in the order *Lactobacillales*. *Lactococcus* includes twelve species and six subspecies (www.bacterio.cict.fr; as of November 2017). *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* ssp. *cremoris* mainly constitute the microbiota of raw milk, cheese and other unheated dairy products. *Lactococcus lactis* ssp. *lactis* is known as primary starter culture in cheese industry along with *Lactococcus lactis* and *Lactococcus lactis ssp. cremoris*. These species are naturally present in raw milk and added to pasteurized milk to facilitate the commercial manufacture of cheeses. The principal role in cheese making is acidification through the production of L−lactate. They also have important role in flavour formation through conversion of amino acids into flavour compounds by proteolysis, citrate utilization and fat metabolism (Quigley et al., 2013).

2.2.2. Antimicrobial activity of *Lactococcus lactis* subsp. *lactis*

The diverse populations in raw milk produce many antimicrobials including bacteriocins, organic acids and hydrogen peroxide. Of these, some *Lactococcus lactis* strains produce the best characterized bacteriocin, nisin. Nisin is used as

biopreservatives throughout the world due to its universal spectrum of activity. *Lactococcus lacti*s strains isolated from raw milk and raw milk products have potential to synthesize nisin with activity against *L. monocytogenes*, *E. coli* and *Staphylococcus* spp.

Also various bacteriocins were reported to be produced by *Lactococcus lactis* including nisin A and its natural variants Z, Q, U, F, lactococcins A, B, M and Z, lacticin LC14 and 481 (Quintana et al., 2017).

2.2.3. *Lactococcus lactis* subsp. *lactis* in dairy industry

Lactococci naturally predominant in raw milk, resulting acidification of the milk as starter culture. As a consequence, microbiological safety is achieved through inhibition of other bacterial growth and organoleptic features are added. *Lactococcus lactis* species especially are marketed as commercial starters for the majority of cheeses due to its ability of conversion milk protein to flavor compounds. The proteolytic system in Lactococci contributes to provide amino acids, the key precursors for essential cheese flavor with diverse protein-hydrolysing enzymes. Bacteriocin-producing lactococcal strains also act a role as useful starters in cheese manufacture improving organoleptic quality by inhibition undesirable flavor or texture induced by spoilage bacteria (Wouters et al., 2002).

2.3. Protective culture

Fermentation is one of the oldest way to preserve food maintaining quality from the time of manufacture to the time of consumption. It is a process based on biological activity of microorganisms for production of diverse metabolites (Ross et al., 2002), one concept included in biopreservation. Although chemical preservation technique as addition of preservatives or physical preservation techniques as heat treatment, high pressure treatment, pulsed electrical field (PEF) and radiation treatment are acknowledged to be useful and powerful, they have limitations in aspects of non-specific bacterial elimination. On the other hand, biopreservation has ability to conserve natural microflora of food (Table 1)(Devlieghere et al., 2004). By empirical knowledge, beneficial microorganisms were preferred to inhibit spoilage and pathogenic bacteria. Notably, LAB are used as starter or adjunct cultures in fermentation for millennia, having status of 'GRAS' (Generally recognized as safe). Metabolism of LAB contributes to control of spoilage and pathogenic bacteria, shelf life without quality change (Holzapfel et al., 1995).

Table 1. Comparison of the food preservation techniques.

Type	Treatment	Advantage	Limitations
Chemical	Preservatives	Multifunction	Safety issues (i.e. adverse effect)
Physical	Heat treatment	Cost-effective	Undesirable organoleptic and , nutritional changes
	High pressure treatment	Minimize organoleptic, nutritional change	Resistant vegetative bacteria, Cost
	Pulsed electrical fields (PEF)		
	Radiation treatment		Consumer resistance
Biological	Biopreservation (Fermentation)	Conserve natural microflora	Interaction with food ingredient

2.3.1. Definition and classification of protective culture

Generally, starter cultures are main fermentation agents that bring about advanced metabolic and sensory changes of food, accompanied by a preservation effect. Although protective cultures are not certainly distinctive concept from starter culture, its main role is in preservation. Protective cultures should improve microbiological safety of food without change of sensory quality of the products. Protective cultures can refer cultures, bacteriocins and fermentates containing such as organic acids, bacteriocin like inhibitory substances or food-grade enzymes.

LAB have special potential for selection and implementation

as protective cultures. The mechanisms of antagonism include competition for nutrients with undesired microorganisms and production of antimicrobial metabolites (Holzapfel et al., 1995).

The effectiveness of protective cultures has been validated in recent several studies over the wide range of food (Bredholt et al., 2001, Budde et al., 2003, Furtado et al., 2015).

2.3.2. Industrial value of protective culture

Globally, 20 % of dairy products are wasted every year in private households, retailers and dairy manufacturers. One solution for reducing waste is extension of shelf life (Westergaard-Kabelmann and Olsen, 2016). Also, consumers' preference to minimally processed foods exception of chemical preservatives and 'mild' products with less acid, sugar or fat contents is increasing(Holzapfel et al., 1995).

In consideration of alternatives to chemical preservatives, LAB and its bioactive compounds are available for protective culture commercially (Varsha and Nampoothiri, 2016). MicroGARD™ (Danisco DuPont) is skim milk fermented by *Propionibacterium freudenreichii* ssp. *shermanii* or specific Lactococci and also contains a heat-resistant protease-sensitive peptides of 700 Da as well as other metabolites. Other products of Danisco DuPont, HOLDBAC® is dairy protective

cultures containing *P. freudenreichii* ssp*. shermanii, L. rhamnosus and L. paracasei* and Nisaplin™ is form of nisin applied to a wide range of foods. The Chr. Hansen developed protective culture, FreshQ® including *L. rhamnosus* and *L. paracasei* (Fernandez et al., 2017). Sacco marketed three types of products for protection, Lyofast. Lyofast products consist of *Carnobacterium* ssp., *L. casei*, *L. rhamnosus, L. plantarum* for inhibition of *Listeria* spp., *Clostridium* spp., yeast and mold (Table 1) (Jones et al., 2005, Varsha and Nampoothiri, 2016).

Table 2. Commercial biopreservation products for dairy products.

Product name (Producer)	Classification	Feature Composition	Target	Application area	Reference
MicroGARD™ (Dupont, USA)	Fermentates	Bacteriocin-like inhibitory products by fermentation of skim milk or dextrose with *Propionibacterium freudenreichii* subsp. *shermanii* or specific Lactococci	Gram negative & positive bacteria, yeast and mold	Wide range of food	
HOLDBAC® (Dupont, USA)	Dairy culture	*L. rhamnosus* *L. paracasei*, *P. freudenreichii* subsp. *shermanii*	Yeast, mold, *Listeria* spp. and some heterofermentative lactic acid bacteria	Fermented foods and cheese	Fernandez et al. (2017)
FreshQ® (Chr.Hansen, Denmark)	Dairy culture	*L. rhamnosus*, *L. paracasei*	Yeast and mold	Fermented dairy products	
Lyofast (Sacco, Italy)	Dairy culture	*Carnobacterium* ssp, *L. casei*, *L. rhamnosus, L. plantarum*	*Listeria* spp., *Clostridium* spp., yeast and mold	Fermented milk products and cheese	Varsha and Nampoothiri (2016)
Nisaplin™ (Dupont, USA)	Antimicrobials	Nisin, a natural bacteriocin produced by fermentation of *Lactococcus lactis*	Gram positive spoilage and pathogenic bacteria	Wide range of food	Jones et al. (2005)

2.3.3. *Lactococcus lactis* subsp. *lactis* as biopreservatives

LAB can produce various antimicrobial substances. Of these, bacteriocin is proteinaceous compound which has bactericidal activity against closely related microorganisms with producers. Bacteriocins can inhibit the growth of spoilage and pathogenic organisms in food. Nisin is the well-known bacteriocin of *Lactococcus lactis*, other bacteriocins and unidentified bacteriocin-like inhibitory substances were found (Wouters et al., 2002).

Bacteriocinogenic lactococcal strains have been used in starter cultures for cheesemaking. The ability of bacteriocin-producing *Lactococcus lactis* to control undesired microorganisms in cheese has been numerously validated. Not only nisin-producing cultures but also other bacteriocinogenic cultures including lacticin 3147 , lacticin 481 and pediocin inhibit *L. monocytogenes* in different type of cheeses (Rodrıguez et al., 2005).

Chapter 3. Materials and methods

3.1. Selection of LAB with antimicrobial activity

3.1.1. Raw milk sampling

Raw milk samples were collected from Hoengseong and Pyeongchang representative 10 farms, respectively (Table 3). Every farms keeps milk refrigerated below 4 ℃ operating bulk tanks. Raw milk was collected with sterile 500 ml bottles from bulk tanks of selected farms. The samples were transported to laboratory on ice within 6 h and kept at 4 ℃ until experiments.

3.1.2. Isolation of LAB

Samples (1 ml) were transferred into sterile 9 ml of 0.85 % NaCl solution (Sigma, USA). Further decimal dilutions were carried out and aliquots were plated on plate count agar containing BromoCresol Purple (BCP) (Eiken chemical Co., Ltd, Japan) and de Man, Rogosa and Sharp (MRS) (Difco, USA) agar supplemented with vancomycin (20 mg/L) (Sigma, USA) and titrated to pH 5. The plates were then incubated at 30 ℃ and 37 ℃ for 24 h under aerobic conditions, respectively. Single colonies with distinct morphologies were picked and inoculated to Tryptic Soy broth (TSB) (Difco, USA) and MRS broth fluid. After incubation, the culture fluids were streaked to confirm purity. Catalase negative and gram positive isolates were

selected and stored at −80 ℃ as a glycerol stock.

Table 3. Origin of the samples for LAB isolation in this study.

Region	Sample	Herd size	Milk production (kg/day)
Hoengseong	1	45	1200
	2	100	3000
	3	45	1200
	4	55	1500
	5	40	1100
	6	60	1500
	7	20	500
	8	25	700
	9	35	1000
	10	80	2500
Pyeongchang	11	60	1500
	12	55	1500
	13	35	1000
	14	80	2200
	15	30	970
	16	64	1950
	17	25	800
	18	39	1200
	19	67	2300
	20	26	780

3.1.3. Antimicrobial activity

For screening of lactic acid bacteria (LAB) with antimicrobial activity, each isolates were examined for antibacterial activity against 7 psychrotrophic spoilage and 7 pathogenic bacteria (except *C. jejuni*) (Table 3) by paper disc diffusion assay described by (Heatley, 1944). Three different methods for evaluating antimicrobial activity were carried out to confirm of reproducibility and standardization of antibacterial effect.

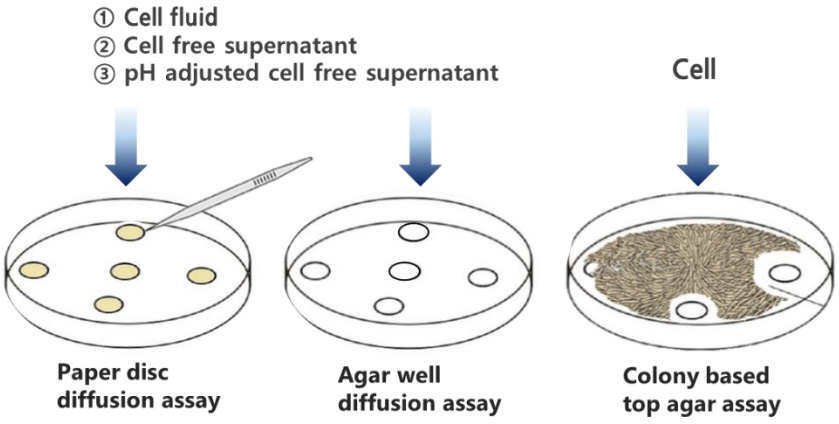

Figure 1. Summary of three antimicrobial activity assays

Table 4. Bacterial strains used as indicators in this study.

Classification	Indicator organisms	Source	Culture medium	Incubation temperature(℃)
Psychrotrophic spoilage bacteria	*Bacillus circulans* KCTC3347	KCTC	NB	30
	Bacillus licheniformis KCTC1918	KCTC	NB	37
	Bacillus subtilis KCTC3135	KCTC	NB	30
	Burkholderia cepacia KCTC2966	KCTC	NB	30
	Micrococcus luteus KCTC3063	KCTC	NB	30
	Pseudomonas fluorescens KCTC42821	KCTC	NB	30
	Pseudomonas putida KCTC1644	KCTC	NB	30
Pathogenic bacteria	*Bacillus cereus* ATCC 14579	KCTC	BHI	30
	Escherichia coli KCTC1682	KCTC	BHI	37
	Listeria monocytogenes KVCC-BA0001449	KVCC	BHI	37
	Listeria innocua KCTC3586	KCTC	BHI	37
	Staphylococcus aureus KCTC 1621	KCTC	BHI	37
	Salmonella typhimurium KVCC-BA0400600	KVCC	BHI	37
	Yersinia enterocolitica wild type	W.T	BHI	37
	Campylobacter jejuni KCTC5327	KCTC	BHI	37

KCTC, Korean Collection for Type Cultures ; KVCC, Korea Veterinary Culture Collection, NB, Nutrient broth; BHI, Brain heart infusion broth.

3.1.3.1. Paper disc diffusion assay

Overnight cultured LAB and indicators were prepared. 100 μl of pathogen suspensions adjusted to OD_{600} (optical density at 600 nm) of 0.5 were spread onto the MRS agar plate mixed with Nutrient agar (NA) (Difco, USA) or Brain Heart Infusion agar (BHI) (Difco, USA) with sterile cotton swabs respectively and then sterile paper discs (6 mm in diameter) were added. The plates were incubated at 30 ℃ for 24 h and diameters of the inhibitory zones were measured in millimetres.

3.1.3.2. Agar well diffusion assay

According to the method of (Balouiri et al., 2016), antimicrobial activity was measured. Overnight cultured LAB and indicators were prepared. A layer of 4 ml of Nutrient broth (NB) or BHI supplemented with 0.8 % (w/v) agarose including 100 μl of pathogen suspensions adjusted to OD_{600} of 0.2 was poured on Tryptic soy agar (TSA) plate. Following drying, wells were punched on the plates using sterile tool. The wells were filled with 100 μl cell fluids, cell free supernatant and pH adjusted cell free supernatant. The plates were incubated at 30 ℃ for 12 h, and diameters of the clearing zones were measured.

3.1.3.3. Colony based top agar assay

Overnight cultured LAB and pathogens were prepared. LAB were inoculated into new MRS broth (0.01 %) and incubated at 30 ℃ for 30 min. Following incubation, the cultures were serially tenfold diluted with 0.85 % NaCl to form approximately 10-20 colonies per plate, spread on an MRS agar plate and incubated at 30 ℃ for 2 h. A layer of 2 ml of top agar, TSB supplemented with 0.5 % (w/v) agarose, was poured on the plates to cover the LAB and incubated for 1 h. Following incubation, a second layer of 3 ml of top agar, NA or BHI agar including 30 μl of pathogen suspensions adjusted to OD_{600} of 0.5 was poured on top of the first layer. The plates were incubated at 30 ℃ for 24 h and ratio of diameters of the zones of clearing and of the colonies were measured.

3.1.4. 16 S rRNA sequencing analysis

The isolated strains were identified using the 16 S rRNA sequence analysis. The 16 S rRNA gene of the strains was amplified using PCR with universal primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The PCR products were sequenced at Macrogen Corporation (South Korea) and DNA sequences were identified by comparison with the BLAST Gene database (http://www.ncbi.nlm.nih.gov).

3.2. Characterization and identification of the selected strain

3.2.1. API test

The selected strain was assayed for carbohydrate fermentation and enzyme production using API 50 CH kit (Bio-Merieux, France) and API ZYM system (Bio-Merieux, France) respectively according to manufacturer's instructions. The API 50 CH test strips were inoculated with overnight cultures and incubated at 30 ℃ for 48 h. For API ZYM system, overnight culture of strains were washed twice with Phosphate-buffered saline (PBS) (Mediatech, Inc., USA) and then adjusted to OD_{600} of 0.1. The suspension was inoculated into API ZYM test strips and incubated at 30 ℃ for 4 h. Results of carbohydrate fermentation and enzymatic activity were recorded in form of color intensity based on the manufacturer's color chart. Additionally, the result was compared to those of the reference strain *Lactococcus lactis* subsp. *lactis* IL1403 (*L. lactis* IL1403).

3.2.2. Growth curve

0.1% inoculum of the selected strain was cultivated to draw growth curves in M17 (Difco, USA) broth supplemented with 0.5 % Lactose, MRS broth and 10 % sterilized skim milk at 30 ℃. The number of viable (CFU/ml) was counted using M17 or MRS agar. The pH of medium was also measured using ORION STAR

A211 pH-meter (Thermo Scientific, USA).

3.2.3. Safety assessment

Antibiotic resistances of the selected isolate were determined with minimal inhibitory concentrations (MICs) according to laboratory standards institute guidelines. The MICs of the following antibiotics were tested: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. Bacterial culture was inoculated on LSM [mixture of Iso-sensitest broth (90 %) and MRS broth (10 %)] agar plate with appropriate M.I.C.Evaluator™ strips(Oxoid, UK) and incubated at 30 ℃ for 48 h under aerobic conditions. The MIC values were measured as the lowest concentration of antibiotics where no visible growth was recorded. For the determination of MIC values of kanamycin, streptomycin and chloramphenicol, the antibiotics were prepared as two-fold dilutions in concentrations ranging between 4 to 512 μg/ml. 100 μl of each concentration of antibiotics were added to 100 μl of double-enriched LSM media in each well of 96-well microtiter plates and 10^6 CFU/ml of isolate was inoculated to each well for final concentrations from 2 to 256 μg/ml. MIC values were measured after incubation at 30 ℃ for 24 h. The microbiological cut-off values (mg/L) of

antibiotics were in accordance to the European Food Safety Authority guidelines (EFSA) and to the European Committee on antimicrobial susceptibility testing (EUCAST).

Hemolytic activity of selected isolate was tested on TSA supplemented with 5 % (v/v) sheep blood. The isolated strain was streaked on the agar, followed by aerobic incubation at 30 ℃ for 72h. *Staphylococcus aureus* ATCC 25923 was used as positive control. Hemolytic activity was detected by appearance of clear zone.

3.2.4. Whole genome sequencing and its analysis

The genomic DNA of *Lactococcus lactis* subsp. *lactis* LDTM 6804 (*L. lactis* LDTM6804) was extracted using FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to manufacturer's instruction and sequenced using a PacBio RSⅡ (Pacific Biosciences, USA) and Illumina Hiseq 4000 platform (Illumina, USA) at Macrogen (South Korea). Primary *De novo* assembly was performed using HGAP3 software to use only PacBio long reads. After assembly, HiSeq reads are applied for accurate genome sequence by using tool Pilon. Information of protein coding sequence, tRNA, rRNA genes on each contig are estimated analyzing sequence homology on the basis of databases. Assembled contigs were annotated based on Eggnog database and RAST annotation pipeline.

3.3. Validation of the selected strain in skim milk

3.3.1. Inoculation of milk

Indicator strains *P. fluorescens* KCTC42821, *B. cepacia* KCTC2966, *L. monocytogenes* KVCC-BA0001449 were propagated in NB or TSB at 30 or 37 ℃ for 24 h and subcultured more than twice before the experiment. The *L. lactis* LDTM6804 was incubated at 30 ℃ for 24 h in M17 broth. For inoculation in skim milk, the cells were pelleted by centrifugation at 14000 rpm for 10 min, washed twice, resuspended in PBS and subsequently diluted to give the desired cell number (10^6 CFU/ml). Single indicator pathogen was inoculated in 100 ml of 10% skim milk at approximately 10^6 CFU/ml with or without *L. lactis* LDTM6804 (10^6 CFU/ml) and stored at 4 , 10 , 15 ℃ during 14 days (Table 5) (Mankaï et al., 2009). The reference strain *L. lactis* IL1403 was used as control group.

Table 5. Test groups for co-culture assay in 10 % skim milk.

No.	Pathogen	Combination of strains
Group 1	*P. fluorescens* (Pfl)	Pfl only
		Pfl +*L. lactis* IL1403
		Pfl +*L. lactis* LDTM6804
Group 2	*B. cepacia* (Bcp)	Bcp only
		Bcp +*L. lactis* IL1403
		Bcp +*L. lactis* LDTM6804
Group 3	*L. monocytogenes* (Lmn)	Lmn only
		Lmn +*L. lactis* IL1403
		Lmn +*L. lactis* LDTM6804

3.3.2. Bacterial enumeration

Skim milk were sampled at day 0, 1, 3, 5, 7, 10 and 14. 1 ml sample was gathered and decimal dilutions in sterile 0.85 % NaCl solution was performed. *P. fluorescens* and *B. cepacia* counts were determined on triplicate plates of Pseudomonas isolation agar (Oxoid, UK) with CFC and *L. monocytogenes* on triplicate plates of Oxford agar (Difco, USA) with oxford antimicrobic supplement (Difco, USA). The plates were incubated at 30 ℃ or 37 ℃ for 48 h (Table 6). *L. lactis* LDTM6804 was on triplicate plates of M17 agar and incubated at 30 ℃. The colonies were enumerated and the results were expressed as log CFU/ml.

Table 6. Selective mediums used for enumeration of viable cells in co-culture assay in 10 % skim milk.

Species	Medium name	Producer	Reference
P. fluorescens *B. cepacia*	Pseudomonas agar base (CM0559B) Pseudomonas CFC selective supplement (SR0103E)	Oxoid	Hinton Jr and Ingram (2005)
L. monocytogenes	Oxford agar (222530) Oxford antimicrobic supplement (211763)	Difco	Gulmez and Guven (2003)

3.3.3. pH measurement

The pH of 10 % skim milk samples inoculated with bacteria was measured at day 0, 1, 3, 5, 7, 10 and 14 with ORION STAR A211 pH-meter (Thermo Scientific, USA).

3.3.4. Quantitative real time PCR (qRT-PCR) analysis

Total mRNA from cultured skim milk samples were extracted using TRIzol (TRI reagent). The cDNA synthesis was carried out using the ReverTra Ace qPCR RT Master Mix with gDNA remover kit (TOYOBO, Japan) according to the instructions of manufacturer. Amplification was carried out in a C1000 Touch thermal cycler (Bio-Rad, USA). The reaction condition was as follows: incubation for 15 min at 37 ° C, 50 ° C for 5 min and 5 min at 98 ° C. Synthesized cDNA was stored at -80 ° C for the experiment. Relative expression of *nisZ, nisB, nisP* and 16S rRNA was detected by real-time PCR with TOPreal™ qPCR 2X PreMIX (SYBR Green with high ROX) kit (Ezynomics, Korea). Real-time PCR was initiated by the denaturation step of 10 min at 95 ℃, followed by 55 cycles of amplification, which were performed according to the following thermal cycling protocol, denaturation for 20 s at 95 ℃, annealing 10 s at 58 ℃ and extension for 30 s at 72 ℃. 16S rRNA gene was used as an endogenous control to normalize the expression of target transcripts. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Bustin and Mueller, 2005) (Table 7)(Figure 2).

Table 7. Primers used in this study.

Primer	Target gene	5'-sequence-3'	Product Size (bp)	TM(℃)	%GC	Reference
nisZ	Nisin structural gene	TGGATTTGGTATCTGTTTCGAAGA	144	57.2	37	Modification (Trmcic,A *et al*, 2011; Ghrairi *et al*, 2004)
		TTACGTGAATACTACAATTACAAGTTGCT		58.9	31	
nisB	Nisin biosynthesis protein	GGGAGAGTTGCCGATGTTGT	131	59.4	55	Modification (Trmcic,A *et al*, 2011)
		TAAAGCCACTCGTTAAAGGGCAGT		61.6	45	
nisP	Nisin leader peptide-processing serine protease	TGCACCGAGTGTATTTGAGGATGT	105	61.4	45	Trmcic,A *et al*, 2011
		GCAGGAGCATAAATTGCATCCG		59.6	50	
341F	16S rRNA	CCTACGGGAGGCAGCAG	450	61.9	70	Shunsuke Takahashi et al., 2014
R806		GGACTACHVGGGTWTCTAAT		50.4	47	

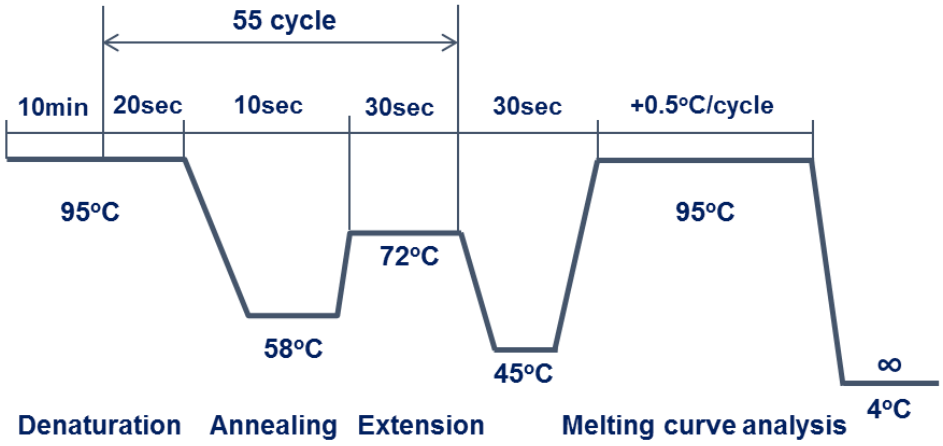

Figure 2. PCR conditions for amplification of nisin genes.

3.4. Application of the selected strain in cheese

3.4.1. Manufacture of cheese

Cow’s raw milk was obtained from Seoul National University farm (Pyeongchang, Gangwon, South Korea) and analyzed by Milkoscan FT2 (Foss Analytical A/S, Denmark) (Table 8). Miniature model cheeses were manufactured based on method of *Hynes et al. (2000)* with modification. The milk was pasteurized at 68 ℃ for 25 min in water bath (SB-22; SciLab, Korea) and then starter strains with and without *L. lactis* LDTM6804 were inoculated to the milk (1 %). LAB culture were inoculated into sterile 10 % skim milk and incubated at 30 ℃ for 24 h before experiment. About 10 min after the addition of the starter culture, the cheese milk was also inoculated with about 10^5 CFU/ml of the indicators. Filtered rennet (0.2 g/L; Chr.Hansen) was then added to the milk and incubated at 32 ℃. After coagulum was sufficiently firm, the coagulum was cut and agitated for 20 min on mechanical stirrer (10 rpm). Whey was drained off and curds were washed with sterile water. Whey removal was continuously carried out by centrifugation. The cheeses were stored at 10 ℃ inside sterile containers during 25 days (Figure 3).

11 different group of cheeses were prepared (Table 9).

Table 8. Gross composition of raw milk used for making cheese.

Constituents	Contents (%)
Total solids (TS)	13.05
Solids non fat (SNF)	8.85
Fat	4.24
Protein	3.16
Lactose	4.56

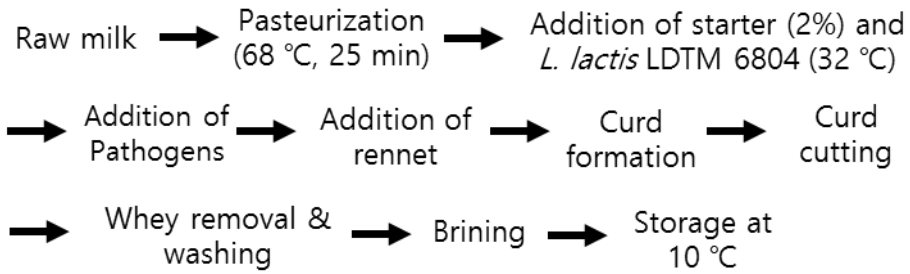

Figure 3. Protocol of miniature cheese model manufacture.

Table 9. Combinations of starter cultures inoculated to miniature cheese control and test groups and their inoculum ratios.

Group		Combination of strains	Ratio
Control 1		*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802	7:3
Control 2		*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6804	7:3
Test 1 (*L. monocytogenes*)	1-1	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802	7:3
	1-2	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802, *L. lactis* subsp. *lactis* LDTM 6804	7:1.5:1.5
	1-3	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6804	7:3
Test 2 (*S. aureus*)	2-1	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802	7:3
	2-2	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802, *L. lactis* subsp. *lactis* LDTM 6804	7:1.5:1.5
	2-3	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6804	7:3
Test 3 (*E. coli*)	3-1	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802	7:3
	3-2	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6802, *L. lactis* subsp. *lactis* LDTM 6804	7:1.5:1.5
	3-3	*L. lactis* subsp. *cremoris* LDTM 6803, *L. lactis* subsp. *lactis* LDTM 6804	7:3

3.4.2. Bacterial enumeration

Cheeses were sampled at day 0, 0.5, 1, 7, 14 and 25. 1 g of sample was gathered and decimal dilutions in sterile 0.85 % NaCl solution was performed. *L. monocytogenes* counts were determined on triplicate plates of Oxford agar (Difco, USA) with oxford antimicrobic supplement (Difco, USA), *S.aureus* on triplicate plates of Mannitol salt agar (Difco, USA) and *E. coli* on triplicate plates of Eosin methylene blue agar (Oxoid, UK). The plates were incubated at 37 ℃ for 48 h (Table 10). The colonies were enumerated and the results were expressed as log CFU/g.

Table 10. Selective mediums used for enumeration of viable cells in cheese.

Species	Medium name	Producer	Reference
L. monocytogenes	Oxford agar (222530) Oxford antimicrobic supplement (211763)	Difco	Gulmez and Guven (2003)
S. aureus	Mannitol salt agar (211407)	Difco	Guilhermetti et al. (2001)
E. coli	Eosin methylene blue agar (CM0069B)	Oxoid	Leininger et al. (2001)

3.4.3. Electronic nose analysis

The flavor compounds were assayed using a Heracles Ⅱ Electronic Nose system produced by Alpha M.O.S. (France), equipped with a Flash Gas chromatograph (two capillary chromatographic columns in parallel) and Combi PAL Auto-sampler System (CTC Analytics AG, Switzerland). The two columns have different polarities (DB-5; apolar and DB-WAX; slightly polar). For calibration before running, Kovats standard was injected into the apparatus and retention indices were calculated based on AroChemBase module in Alpha Soft software. 1 g of each cheese was put and capped in 20 ml vials. The headspace was generated for 20 min at 70 ℃ with agitation at 500 rpm. The volatiles were then transferred through syringe to an inlets injector where compounds flash evaporate. After condensation of sample gas compounds in absorbent trap and flash heating process, the volatiles were delivered rapidly to two capillary chromatographic columns. The column temperature increased from 50 ℃ (initial, hold time 2 s) up to 250 ℃ (21 s) with an increment of 1 ℃/s to 80 ℃ and an increment of 3 ℃/s to 250 ℃. The two flame ionization detectors (FID) working in parallel and the data was recorded for analysis by specialized software (AroChemBase) using data processing techniques as Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA). Both PCA score plot and DFA model were

constructed with the most discriminant peaks selected as representative odorants that contribute to the discrimination of cheese samples.

3.5. Statistical analysis

All data were statistically analyzed using Graph Pad Prism software Version 5.01 (Graph Pad Software, USA). The statistical significance of differences was determined by the Tukey's post hoc test. Differences were considered significant at $p < 0.05$.

Chapter 4. Results

4.1. Selection of LAB with antimicrobial activity

4.1.1. Isolation of LAB

306 distinct isolates were obtained from 20 raw milk samples using selective medium and differential medium. The 186 isolates were acquired using MRS agar supplemented with vancomycin titrated to pH 5 and 120 isolates were BCP agar. Paper disc diffusion assay was performed with all isolates against 7 pathogenic bacteria and 7 psychrotrophic bacteria. Of these, 5 isolates were selected based on criteria of range of spectrum. The isolates were further identified as *Lactococus lactis*, *Lactobacillus casei, Lactobacillus plantarum*, *Lactobacillus paracasei*, *Pediococcus acidilactici* by 16S rRNA sequencing and BLAST search.

One isolate *Lactococcus lactis* subsp. *lactis* LDTM 6804 (*L. lactis* LDTM6804) with the high antimicrobial activity and broad spectrum was finally selected (Figure 4).

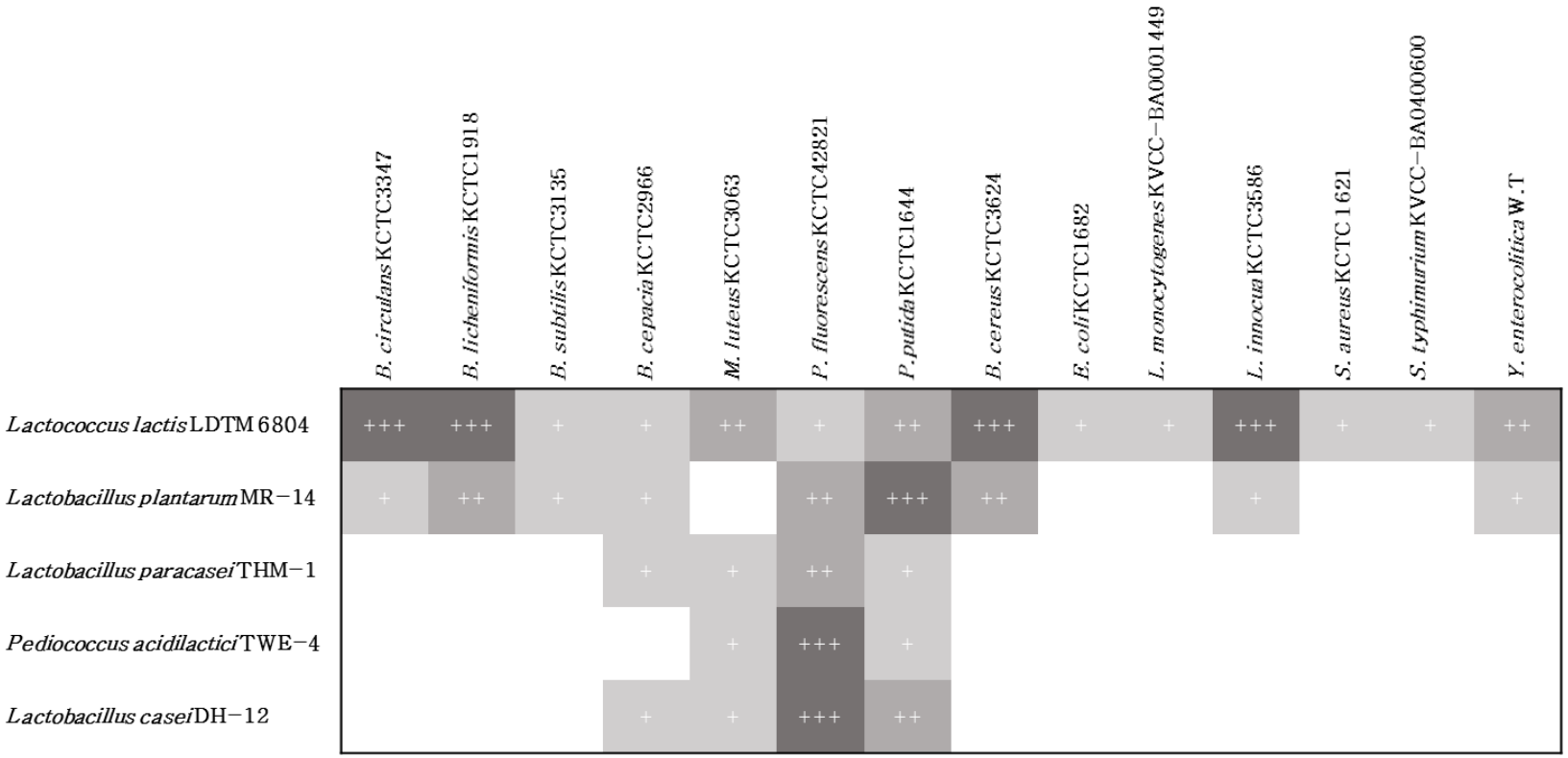

Figure 4. Antimicrobial activity of LAB against indicators. +++, >10.6 mm ; ++, >9.3 mm ; +, >8 mm.

4.1.2. Antimicrobial activity

The antimicrobial activity of screened isolate *L. lactis* LDTM6804 was confirmed using three different methods, paper disc diffusion assay, agar well diffusion assay and colony based top agar assay against psychrotrophic spoilage and pathogenic bacteria (Table 12).

Results were consistent in three assays except antimicrobial activity of cell free supernatant against *B. circulans*, *B. cepacia* and *S. typhimurium*. The cell free supernatant of *L. lactis* LDTM6804 showed inhibition zones by an agar well diffusion assay. No inhibition was observed with same supernatants by paper disc diffusion assay. The results of the agar well diffusion assay demonstrated the antagonistic activities of *L. lactis* LDTM6804 has against every indicator strains tested in this experiment.

Table 11. Antimicrobial activity of *L. lactis* LDTM 6804 by 3 different assays.

		Paper disc assay			Agar well diffusion assay			Top agar assay
		cell	cell free supernatant	pH adjusted supernatant	cell	cell free supernatant	pH adjusted supernatant	cell
Psychrotrophic spoilage bacteria	*B.circulans*	+	−	−	+	+	−	+
	B.licheniformis	+	+	+	+	+	+	+
	B.subtilis	+	+	+	+	+	+	+
	B.cepacia	+	−	−	+	+	−	+
	M.luteus	+	+	+	+	+	+	+
	P.fluorescens	+	+	−	+	+	−	−
	P.putida	+	+	+	+	+	+	−
Pathogenic bacteria	*B.cereus*	+	−	−	+	−	−	+
	E.coli	+	−	−	+	−	−	+
	L.innocua	+	+	+	+	+	+	+
	L.monocytogenes	+	+	+	+	+	+	+
	S.typhimurium	+	−	−	+	+	−	+
	S.aureus	+	+	+	+	+	+	+
	Y.enterocolitica	+	−	−	+	−	−	+
	C.jejuni	−	−	−	+	+	+	−

4.2. Characterization and identification of the selected strain

4.2.1. Biochemical property

The *L. lactis* LDTM6804 showed slightly different sugar fermentation patterns and enzymatic profile compared to the reference strain *L. lactis* subsp. *lactis* IL1403 (*L. lactis* IL1403) (Figure 5).

4.2.2. Growth curve

The *L. lactis* LDTM6804 showed similar growth kinetics in different type of medium, M17, MRS and 10 % skim milk. The maximum growth was reached at 24 h and the number of viable cell was $5x10^9$CFU/ml in M17 medium. (Figure 6).

4.2.3. Safety assessment

The MIC values of *L. lactis* LDTM6804 was measured for antibiotics. In accordance with the biological breakpoints, the selected strain was found to be susceptible to all of the antibiotics tested. Hemolytic activity was not exhibited (Table 13).

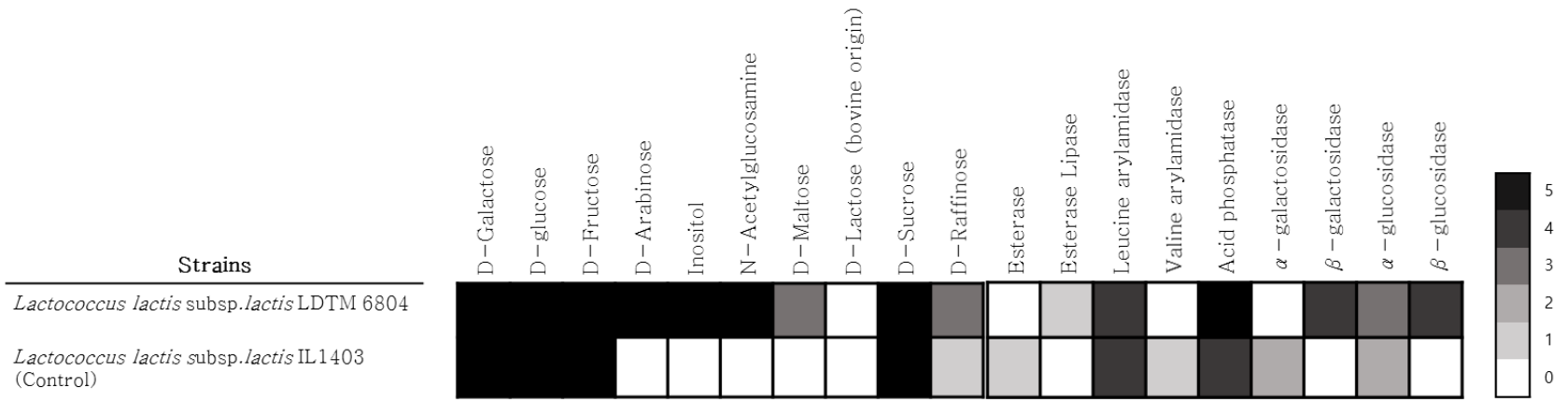

Figure 5. Sugar fermentation and enzymatic profiling of isolated strains by API CH50 kit and API ZYM kit (BioMerieux, France).

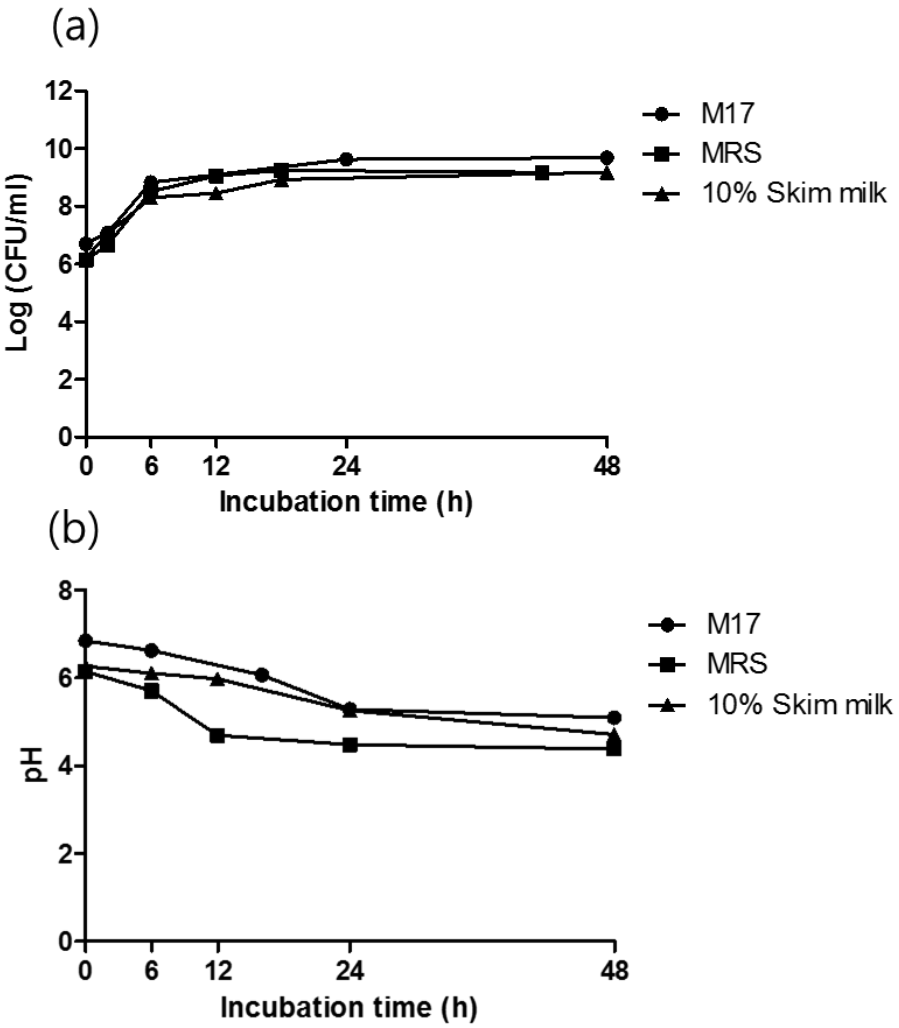

Figure 6. Growth and pH changes of *L. lactis* LDTM6804 in M17, MRS and 10 % skim milk media at 30 ℃ (a) Viable cell counts; (b) pH.

Table 12. Susceptibility to antibiotics and hemolytic activity of *L. lactis* LDTM6804.

Species	Strains	Susceptibilities to the following antibiotics (MIC [μg ml^{-1}])									Hemolytic activity (+/−)
		AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL	
Lactococcus lactis. lactis	LDTM6804	0.12	0.5	16	2	8	0.12	0.12	0.12	8	−
Suggested bacterial cut−off values in accordance to the European Food Safety Authority (EFSA)											
Lactococcus lactis		2	4	32	64	32	1	1	4	8	
Suggested bacterial cut−off values in accordance to the European Committee on antimicrobial susceptibility testing (EUCAST)											
Gram positive anaerobe		4	2	−	−	−	−	4	−	8	

AMP, VAN, GEN, KAN, STR, ERY, CLI, TET, CHL and refer to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol respectively
**Staphylococcus aureus* ATCC 25923 used as positive control

4.2.4. Genomic analysis

Draft genome of *L. lactis* LDTM6804 was obtained, which was 2,490,563 bp in size and possessed a GC contents of 35.02 %. Two contigs were assembled and complete genome sequence of *L. lactis* LDTM6804 shows a single circular chromosome of 2,490,563 bp with 2,409 of coding sequences (CDS), 63 of tRNA and 19 of rRNA genes.

The software program RAST was used to identify protein-coding genes and gene annotation.

A comparative analysis of *L. lactis* LDTM6804 and reference strain *L. lactis* IL1403 was performed. This comparative genomics were analyzed with genes involved in antimicrobial ability.

Table 13. Summary of assembly of *L. lactis* LDTM6804.

Contigs[a]	Total length[b]	N50[c]	Max length[d]	Min length[e]	Average length[f]
2	2,490,563	2,417,707	2,417,707	72,856	1,245,281

[a] The number of contigs assembled.
[b] The total number of bases in the contigs.
[c] Half of all bases reside in contigs of this size of longer.
[d] The sequence size of the longest contigs.
[e] The sequence size of the shortest contigs.
[f] The average contigs size.

Table 14. Summary of genome annotation of *L. lactis* LDTM6804 and *L. lactis* IL1403.

Strain	Base (bp)	Gene	GC contents (%)	CDS	tRNA	rRNA	ANIb[a] (%)	Reference
L. lactis subsp. *lactis* IL1403	2,365,589	2,310	35.4	1,987	62	6	–	Bolotin A, *et al.*, 2001
L. lactis subsp. *lactis* LDTM6804	2,490,563	2,492	35.02	2,409	63	19	98.31	This study

[a] Average nucleotide identity based on BLAST to the reference strain IL1403.

The sequence of *L. lactis* LDTM6804 revealed pairwise similarity of 98.31 % with *L. lactis* IL1403.

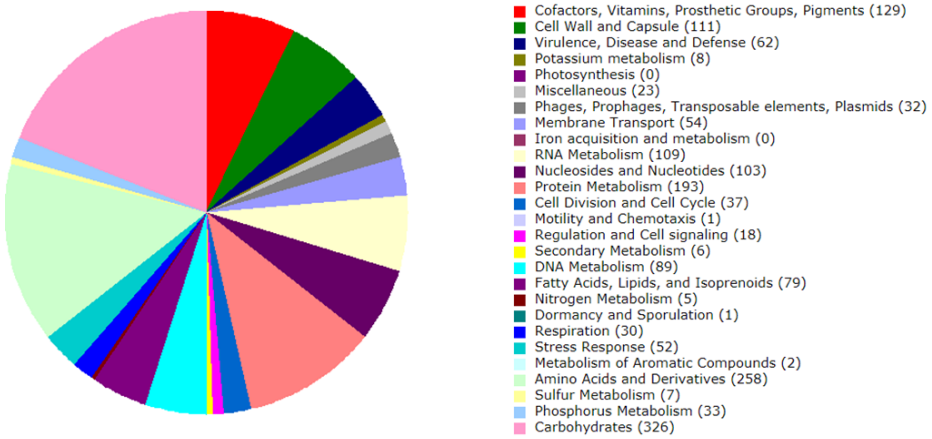

Figure 7. Distribution of genes classified based on subcategory.

Also, gene cluster involved in nisin Z biosynthesis was identified according to the whole genomic analysis of *L. lactis* LDTM6804. Gene cluster is comprised of 11 distinct genes, *nis Z, B, T, C, I, P, R, K, F, E, G.*

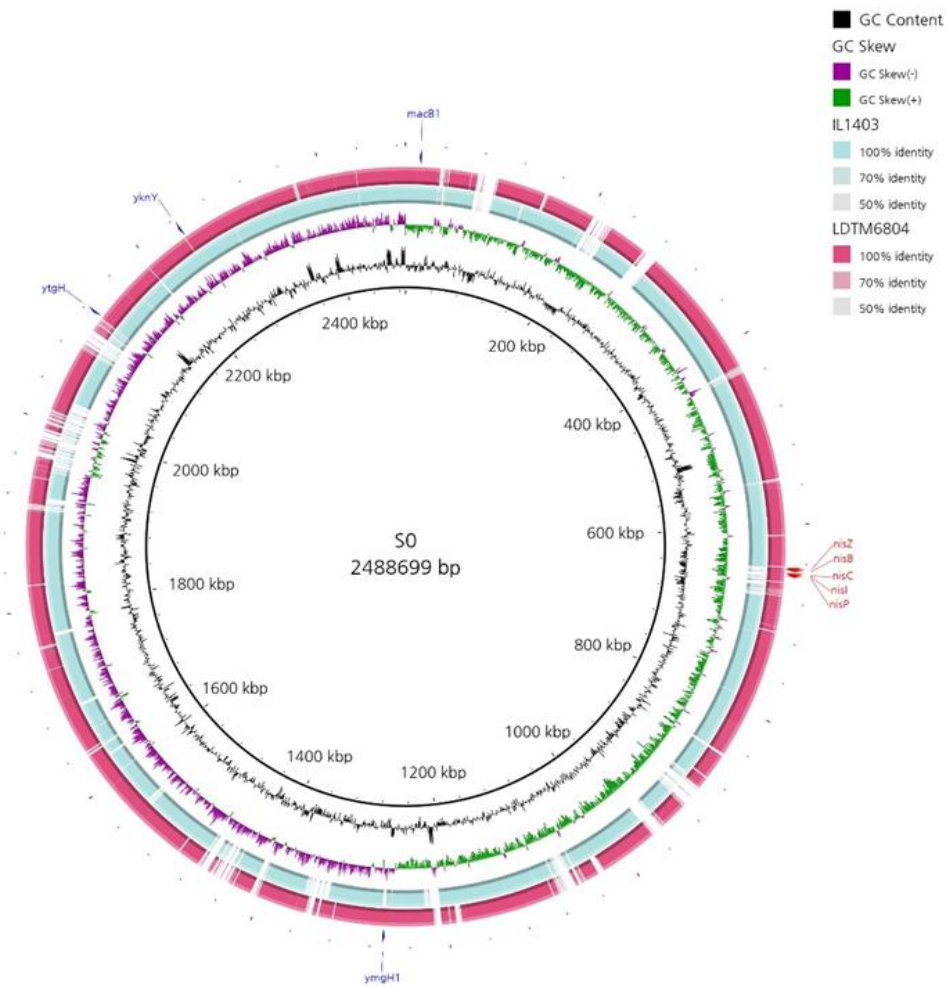

Figure 8. Complete genome sequence analysis and comparison with IL1403 and S0. BRIG images showing genomic regions between IL1403, S0 and LDTM6804. The innermost rings show GC content (black) and GC skew (purple/green). The sky blue ring show BLAST comparison of IL1403 against S0 and pink ring show BLAST comparison of LDTM 6804 against S0.

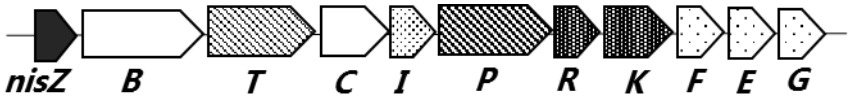

Figure 9. Organization of genes involved in nisin Z production, regulation and immunity.

Table 15. Summary of genes involved in nisin Z synthesis.

Gene	Gene product
nisZ	Nisin structural gene
nisB	Nisin biosynthesis protein
nisT	Nisin transport ATP-binding protein
nisC	Nisin biosynthesis protein
nisI	Nisin immunity protein
nisP	Nisin leader peptide-processing serine protease
nisR	Nisin biosynthesis regulatory protein
nisK	Nisin biosynthesis sensor
nisF	Nisin transport/immunity protein
nisE	
nisG	

4.3. Validation of the selected strain in skim milk

4.3.1. Inhibition of pathogens

In the skim milk experimentally contaminated with 6.69 logCFU/ml of *P. fluorescens* KCTC42821, the log counts of *P. fluorescens* slightly decreased after 14 days under 4 ℃, increased to 8.18 ± 0.06 at 10 ℃ and 9.32 ± 0.03 at 15 ℃. Samples of skim milk inoculated with *L. lactis* LDTM6804, growth of *P.fluorescens* was inhibited and the average counts after 14 days were 2 log units lower than skim milk with nonbacteriocinogenic *L. lactis* IL1403 at 10 ℃. At 15 ℃, the counts were below the detection level after 10 days as shown in Fig 10.

Counts of *B. cepacia* KCTC2966 in inoculated milk were 6.46 logCFU/ml. *B. cepacia* counts were decreased during storage under 4 and 10 ℃. The log counts of *B. cepacia*, on the other hand, increased shortly after decrease in the early 1 day at 15 ℃. The addition of *L. lactis* LDTM6804 resulted in a reduction of *B. cepacia,* with counts 1.29 log units compared with skim milk with nonbacteriocinogenic *L. lactis* IL1403 after 14 days at 10 ℃. Also inhibition was detected at 15 ℃, the counts were below the detection level after 10 days (Fig 11).

The skim milk contaminated with 6.57 logCFU/ml, the log

counts of *L. monocytogenes* KVCC-BA0001449 were 5.35 ± 0.07, 8.30 ± 0.10 and 8.07 ±0.04 after 14days under 4 ℃, 10℃ and 15℃, respectively. In the end of the experiment on day 14, counts of *L. monocytogenes* co-cultivated with *L. lactis* LDTM6804 were 0.6 and 4.93 log units lower than skim milk with nonbacteriocinogenic *L. lactis* IL1403 at 10℃ and 15℃, respectively (Fig 12).

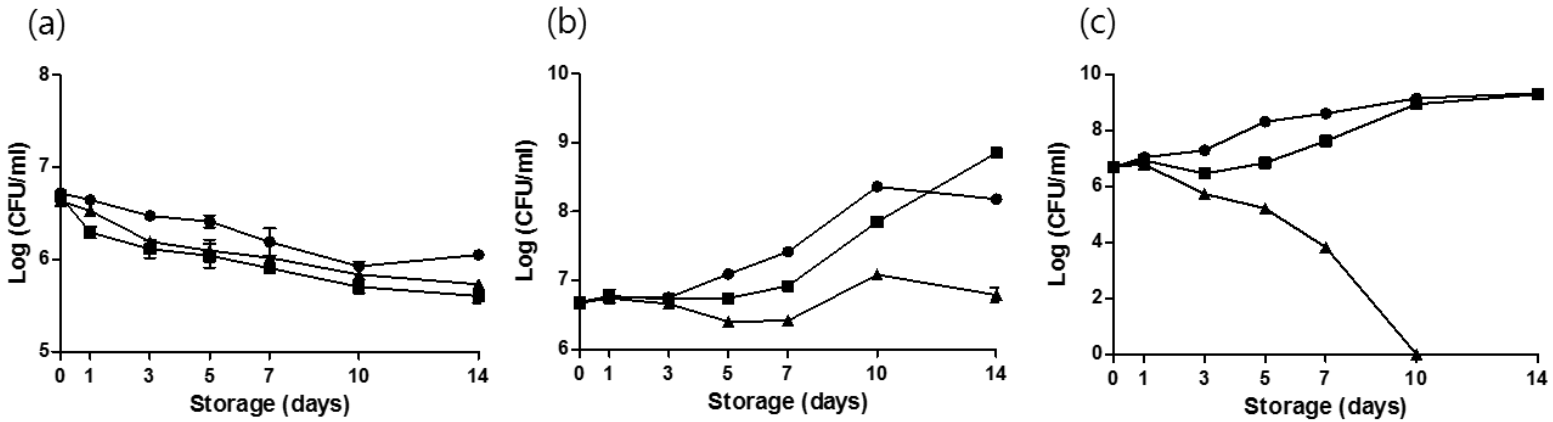

Figure 10. Inhibition of *P. fluorescens* KCTC42821 in skim milk inoculated with nisin producing *L. lactis* LDTM6804 stored at (a) 4 ℃; (b) 10 ℃ and (c) 15 ℃. (●), only *P. fluorescens*; (■), co-cultured with non-bacteriocinogenic IL1403; (▲), co-cultured with LDTM6804 producing nisin.

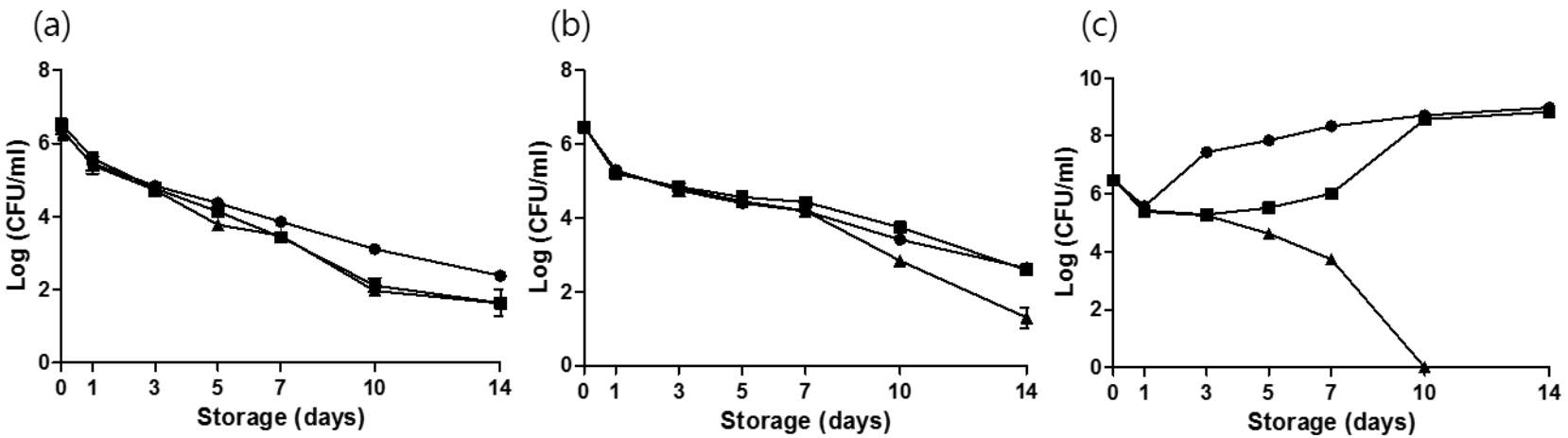

Figure 11. Inhibition of *B. cepacia* KCTC2966 in skim milk inoculated with nisin producing *L. lactis* LDTM6804 stored at (a) 4 ℃; (b) 10 ℃ and (c) 15 ℃. (●), only *B. cepacia*; (■), co-cultured with non-bacteriocinogenic IL1403; (▲), co-cultured with LDTM6804 producing nisin.

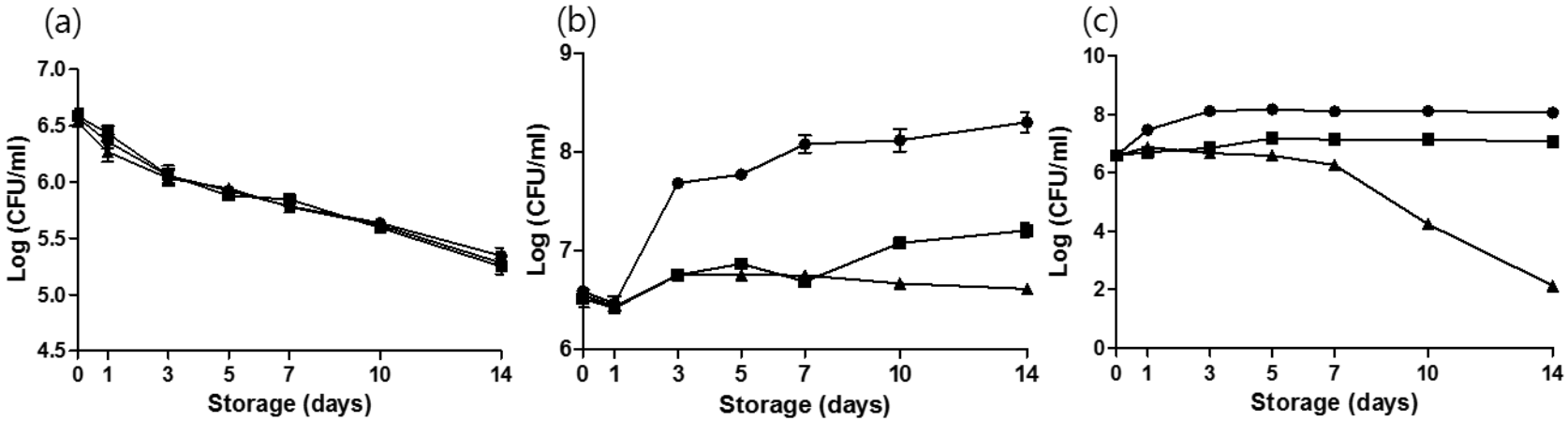

Figure 12. Inhibition of *L. monocytogenes* KVCC-BA0001449 in skim milk inoculated with nisin producing *L. lactis* LDTM6804 stored at (a) 4 ℃; (b) 10 ℃ and (c) 15 ℃. (●), only *L. monocytogenes*; (■), co-cultured with non-bacteriocinogenic IL1403; (▲), co-cultured with LDTM6804 producing nisin.

4.3.2. Expression of nisin encoding gene

Relative expression ratios of *nis Z, B* and *P* genes in skim milks contaminated with pathogens are presented in Figure 13. From these data, expression of the *nis B* and *P* genes showed similar patterns through fermentation. Under 4 ℃, relative expression of genes encoding *nis Z, B* and *P* was low. The expression of *nis Z* was increased gradually in skim milks contaminated with *B. cepacia* and *L. monocytogenes* at 15 ℃.

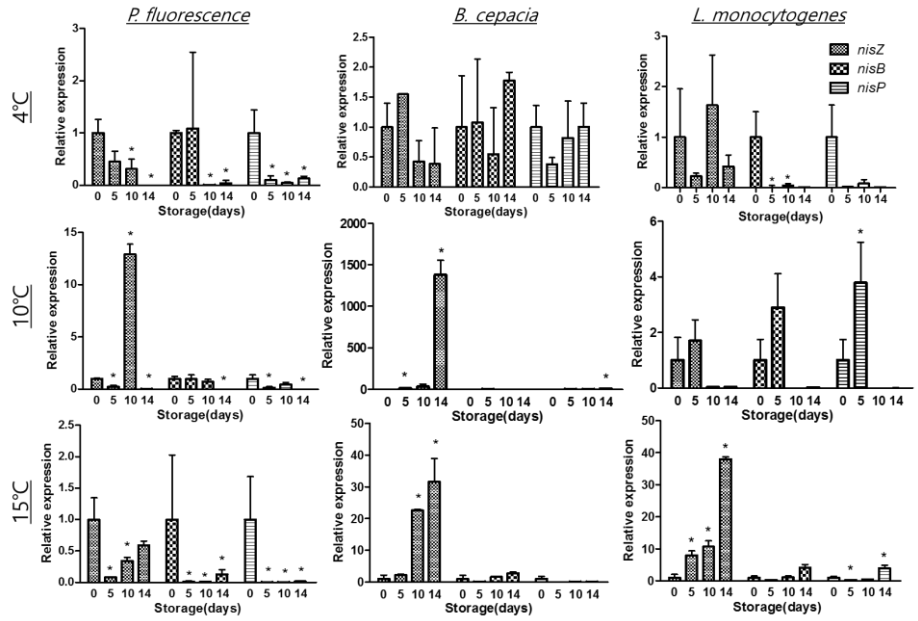

Figure 13. Rates of relative expression of *nis Z*, *B* and *P* in skim milk contaminated with pathogens during storage at 4, 10, 15 ℃ for 0, 5, 10 and 14 days. Significance is indicated as follows: $*p<0.05$

4.4. Application of the selected strain in cheese

4.4.1. Inhibition of pathogens

The survival of *L. monocytogenes* KVCC-BA0001449, *S. aureus* KCTC1621 and *E. coli* KCTC1682 was monitored during cheese manufacture and ripening. Reduced counts of *L. monocytogenes* and *S. aureus* were observed in cheeses containing *L. lactis* LDTM6804 immediately after manufacture.

As shown in Table 16, counts of *L. monocytogenes* in inoculated milk were 5.04 log CFU/ml. In cheese made from milk not inoculated with *L. lactis* LDTM6804, *L.monocytogenes* counts were 4.44 logCFU/g after 3 d, and increased constantly until the end of the experiment. Test groups showed similar change of the cell counts with control cheese. *L.lactis* LDTM6804 inhibited the growth of *L.monocytogenes* 0.15 and 0.42 log units compared to control group at day 25.

The reduction in the *S. aureus* was nearly similar to the *L. monocytogenes*. The populations of *S. aureus* decreased by 0.2 and 0.57 log units in cheese containing *L.lactis* LDTM6804 compared to control group at day 25.

During storage, in the absence of *L.lactis* LDTM6804, *E. coli* grew through constantly, reaching about 10^8 CFU/g. However, *E. coli* counts were reduced in cheese inoculated with *L.lactis* LDTM6804, 0.74 and 0.26 log units compared to control group at day 25.

Table 16. Survival of *L. monocytogenes* KVCC-BA0001449 in cheeses manufactured with a *L. lactis* LDTM6804.

Starters	Storage time (days)						
	0	0.5	1	3	7	14	25
LDTM 6803 + LDTM 6802	5.04±0.02	3.74±0.10	4.74±0.02	4.44±0.04	4.69±0.10	4.59±0.07	5.18±0.02
LDTM 6803 + LDTM 6802 +nisin producing LDTM6804	5.04±0.02	3.73±0.05	4.29±0.02**	4.48±0.05	4.60±0.04	4.40±0.09*	5.03±0.03**
LDTM 6803 +nisin producing LDTM6804	5.04±0.02	4.01±0.03**	4.18±0.02**	4.38±0.04	4.31±0.05**	4.42±0.03*	4.76±0.02**

The data are shown as mean log CFU/g ± standard deviation. Significance is indicated as follows: *p<0.05; **p<0.01

LDTM6803, *Lactococcus lactis* subsp. *cremoris* LDTM6803; LDTM6802, *Lactococcus lactis* subsp. *lactis* LDTM6802; LDTM6804, *Lactococcus lactis* subsp. *lactis* LDTM6804.

Table 17. Survival of *S. aureus* KCTC1621 in cheeses manufactured with a *L. lactis* LDTM6804.

Starters	Storage time (days)						
	0	0.5	1	3	7	14	25
LDTM 6803 + LDTM 6802	4.58±0.02	4.4±0.00	4.59±0.02	4.68±0.03	4.79±0.02	4.67±0.04	4.50±0.02
LDTM 6803 + LDTM 6802 +nisin producing LDTM6804	4.58±0.02	4.22±0.07**	4.34±0.03**	4.42±0.06**	4.53±0.02**	4.43±0.00**	4.30±0.05**
LDTM 6803 +nisin producing LDTM6804	4.58±0.02	3.78±0.00**	3.77±0.10**	3.82±0.10**	4.15±0.05**	4.03±0.04**	3.93±0.00**

The data are shown as mean log CFU/g ± standard deviation. Significance is indicated as follows: $*p<0.05$; $**p<0.01$

Table 18. Survival of *E. coli* KCTC1682 in cheeses manufactured with a *L. lactis* LDTM6804.

Starters	Storage time (days)						
	0	0.5	1	3	7	14	25
LDTM 6803 + LDTM 6802	5.86±0.04	7.09±0.03	7.19±0.01	7.52±0.05	7.59±0.01	8.04±0.02	8.19±0.04
LDTM 6803 + LDTM 6802 +nisin producing LDTM6804	5.86±0.04	6.54±0.07**	7.09±0.07	7.44±0.09	7.28±0.03**	7.14±0.10**	7.45±0.06**
LDTM 6803 +nisin producing LDTM6804	5.86±0.04	6.57±0.05**	6.71±0.01**	7.19±0.06**	7.04±0.00**	7.28±0.04**	7.93±0.03**

The data are shown as mean log CFU/g ± standard deviation. Significance is indicated as follows: $*p<0.05$; $**p<0.01$

4.4.2. Nisin related gene expression analysis

Relative expression ratios of *nis Z, B* and *P* genes in cheeses contaminated with pathogens are presented in Figure 14.

Changes of relative expression ratios of nisin encoding genes challenged with *L. monocytogenes* and *S. aureus* throughout storage were similar. Expression of *nis Z* increased to day 14 and decreased to day 25. However, high amounts of *nis Z* gene expression were observed in the cheese of test 3 group challenged with *E. coli*. Expression of the *nis B* and *P* genes increased gradually through ripening.

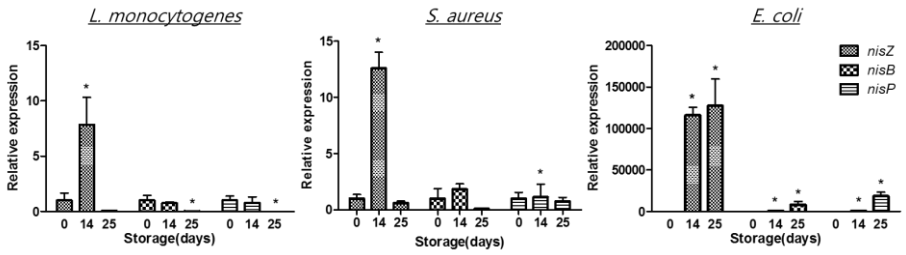

Figure 14. Rates of relative expression of *nis Z*, *B* and *P* in cheese contaminated with pathogens during storage at 10 ℃ for 0, 14 and 25 days. Significance is indicated as follows: $*p<0.05$

4.4.3. Flavor compounds analysis

4.4.3.1. Flavor discrimination

PCA of the raw data was obtained with the electronic nose. The PCA plot given in Fig 15, showed separation of 2 groups.

Figure 16 showed the DFA plot derived from the same data and good differentiation was observed. It was possible to discriminate between control groups and test groups by using the first two discriminant factors (DF1; 70.005 %, DF2; 11.428 %) It was also possible to discriminate the ripening periods by using the first two discriminant factors (DF1; 79.211 %, DF2; 20.789 % (Figure 17).

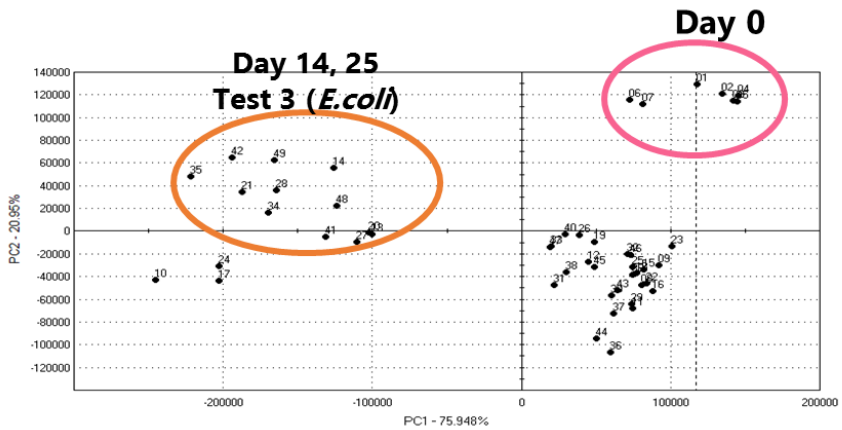

Figure 15. Principal Component Analysis (PCA) for flavor pattern of the cheeses.

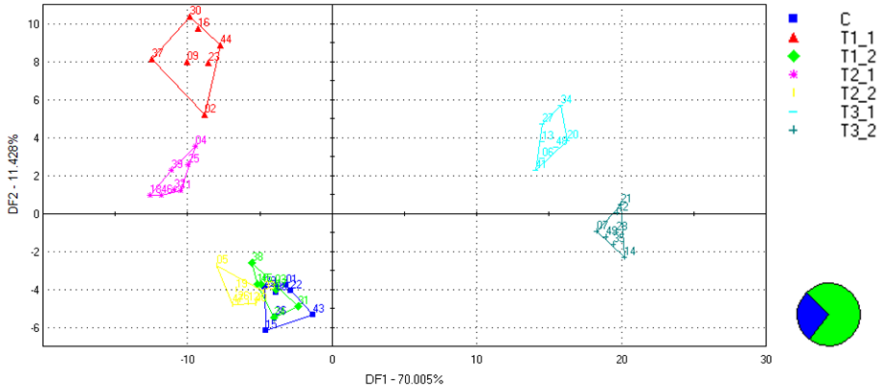

Figure 16. Discriminant Function Analysis (DFA) for flavor pattern of the cheeses between control and test group.

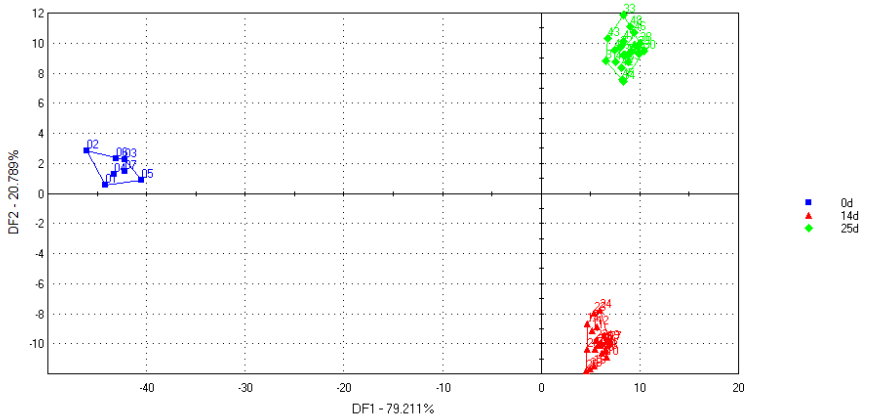

Figure 17. Discriminant Function Analysis (DFA) for flavor pattern of the cheeses on different ripening periods.

4.4.3.2. Flavor compounds analysis

Representative flavor compounds were described with sensory descriptors in Table 19. Test groups without *L. lactis* LDTM6804 have some distinct flavor profile compared to the test groups with *L. lactis* LDTM6804. Most of the flavor compounds increased during the ripening period, for examples, ethanol, 3-

Buten-2-one, acetoin, isopropyl isothiocyanate and formic acid. The flavor 2-nitropropane was formed in Test 1-1, Test 2-1, Test 3-1 and Test 3-2. In the test 3-1 groups, methoxyflurane, 2,4,5-Trimethyl-1,3-dioxolane and butanethiol increased during ripening period. However, trans-4-thujanol and methoxyflurane increased during ripening period in the test 3-2 groups.

Table 19. Flavor profiling and expected sensory descriptors analyzed by electronic nose.

	Chemical Names	Formula	Sensory descriptors	reference
Control group	Ethanol	C_2H_6O	Alcoholic, sweet	Arochembase
	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	Butane-2,3-dione	$C_4H_6O_2$	Butter, Creamy, Fruity	Arochembase
	3,3-Dimethylbutane-2-ol	$C_6H_{14}O$	Camphor	Arochembase
	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	(Duque, Carmenza, *et al.*, 2001)
	Acetoin	$C_4H_8O_2$	Butter, Coffee, Creamy	Arochembase
Test Group 1-1	Ethanol	C_2H_6O	Alcoholic, sweet	Arochembase
	2-Nitropropane	$C_3H_7NO_2$	-	-
	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	Ethanol,2-ethoxy-	$C_4H_{10}O_2$	-	-
	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	(Duque, Carmenza, *et al.*, 2001)
Test Group 1-2	Ethanol	C_2H_6O	Alcoholic, sweet	Arochembase
	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	Ethanol,2-ethoxy-	$C_4H_{10}O_2$	-	-
	Acetoin	$C_4H_8O_2$	Butter, Coffee, Creamy	Arochembase
	3,3-Dimethylbutane-2-ol	$C_6H_{14}O$	Camphor	Arochembase
Test Group 2-1	Ethanol	C_2H_6O	Alcoholic, sweet	Arochembase
	3-Buten-2-one	C_4H_6O	Sweet	Arochembase
	Butane-2,3-dione	$C_4H_6O_2$	Butter, Creamy, Fruity	Arochembase
	2-Nitropropane	$C_3H_7NO_2$	-	-
	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	(Duque, Carmenza, *et al.*, 2001)
Test Group 2-2	Ethanol	C_2H_6O	Alcoholic, sweet	Arochembase
	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	Butane-2,3-dione	$C_4H_6O_2$	Butter, Creamy, Fruity	Arochembase
	Ethanol,2-ethoxy-	$C_4H_{10}O_2$	-	-
	3,3-Dimethylbutane-2-ol	$C_6H_{14}O$	Camphor	Arochembase
Test Group 3-1	Propenal	C_3H_4O	-	-
	Butane-2,3-dione	$C_4H_6O_2$	Butter, Creamy, Fruity	Arochembase
	2,4,5-Trimethyl-1,3-dioxolane	$C_6H_{12}O_2$	-	-
	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	(Duque, Carmenza, *et al.*, 2001)
	Methoxyflurane	$C_3H_4Cl_2F_2O$	-	-
Test Group 3-2	Propenal	C_3H_4O	-	-
	Butane-2,3-dione	$C_4H_6O_2$	Butter, Creamy, Fruity	Arochembase
	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	(Duque, Carmenza, *et al.*, 2001)
	Trans-4-Thujanol	$C_{10}H_{18}O$	-	-

* Database provided by electronic nose manufacturer (Alpha M.O.S, France) which is linked with NIST database (http://webbook.nist.gov/chemistry).

Chapter 5. Discussion

The demands of natural preservatives and minimally processed products are increasing among consumers in food industry (Raichurkar and Athawale, 2015). Recently, there has been significant commercial interest in the use of lactic acid bacteria as natural food preservatives to enhance food safety and stability by producing of antimicrobial compounds (El-Shafei et al., 2008). Therefore, in the present study, we investigated the antimicrobial activity of nisin Z producing *Lactococcus lactis* subsp. *lactis* LDTM6804 (*L.lactis* LDTM6804) against psychrotrophic spoilage and pathogenic bacteria using two dairy product models.

A total of 306 isolates originated from 20 different raw milks were examined for their antimicrobial activities against 14 indicator strains. One isolate showing the potential antibacterial activity was characterized with innate properties and product of bacteriocin. *L.lactis* LDTM6804 was identified as nisin Z producer through whole genome sequence analysis. Nisin Z, natural nisin variant as nisin Q, F and U differs from nisin A only at position 27, where Asn replaces His(Ko et al., 2015, Hwanhlem et al., 2017). Inhibition of food-spoilage and foodborne pathogenic bacteria by nisin Z producing Lactococci has been studied (de Paula et al., 2015, Hwanhlem et al., 2017) .

As previously observed, nisin Z producing *Lactococcus lactis ssp. lactis* isolated from raw milk presented a broad inhibitory activity against several foodborne pathogens(Perin et al., 2013). To verify the antibacterial activity *in situ*, *L.lactis* LDTM6804 was tested against indicator strains in reconstituted skim milk and cheese in this study.

Except for skim milk samples stored at 4 ℃, the growth of *P. fluorescens, B. cepacia, L. monocytogenes* were inhibited when *L.lactis* LDTM6804 was added compared to the control samples without *L.lactis* LDTM6804 at 10 and 15 ℃. Less effects in reducing indicators counts of the treatments with non-bacteriocinogenic strain (*Lactococcus lactis* subsp. *lactis* IL1403) support this inhibitory activity of *L.lactis* LDTM6804 by bacteriocin production. Although *L.lactis* LDTM6804 was not found to rapidly reduce indicators as compared to application of nisin Z preparation in the current study (Mitra et al., 2011), there was no regrowth of indicators detected except for *P. fluorescens* in milk samples co-cultivated with *L. lactis* LDTM6804 after day 7 at 10 ℃. This may suggest that the *L.lactis* LDTM6804 prevent the regrowth of pathogens or development of resistance to the nisin. Also, rapid killing of indicator bacteria can be achieved by increasing the number of viable *L.lactis* LDTM6804. Application to milk provides important data for effects of milk components on antimicrobial strategy as pointed out by the current

studies(Bizani et al., 2008, Furtado et al., 2015). With the above results, *L.lactis* LDTM6804 could be used as a protective culture in dairy products stored at temperature above 10 ℃. Using purified nisin Z or fermentates from *L.lactis* LDTM6804, psychrotrophic spoilage and pathogenic bacteria could be inhibited at refrigerated temperature.

In cheese models, cheeses inoculated with *L.lactis* LDTM6804 achieved slight reductions up to 0.42 log units for *L. monocytogenes*, 0.57 log units for *S. aureus* and 0.74 log units for *E. coli,* respectively after 25 d of ripening in comparison with control cheeses without *L.lactis* LDTM6804. In the case of *L. monocytogenes*, this result could not reflect sufficiently the previous results of antimicrobial activity of *L.lactis* LDTM6804 tested on agar plate and model skim milk. The recent study pointed out that inhibitory activity against pathogens in cheese manufactured with a nisin-producing starter culture could be related with bacteriocin activity or differences in cheese pH (Rodrıguez et al., 2005). In addition, the observed reduction could be sufficient in the case of naturally contaminated milk or dairy product, because the inocula of pathogens were several log units above the levels of actual numbers of undesirable bacteria. The impact of nisin producing *L.lactis* LDTM6804 would presumably be greater than the effect found in the current study (Luukkonen et al., 2005, Rodrıguez et al., 2005). To improve the

antimicrobial activity of nisin producing *L.lactis* LDTM6804, further studies are needed including combinations of other bacteriocin producing microorganisms (Rodrıguez et al., 2005).

To detect *in situ* production and activity of bacteriocin throughout fermentation and ripening, genes involved nisin biosynthesis was evaluated using molecular (quantitative real-time PCR) methods. Bacteriocin activity in food systems is difficult to measure due to the detection in complex environments, therefore, very few studies on time-dependent expression of nisin in food was quantified (Trmčić et al., 2011). The quantification of nisin expression during inhibition against pathogens has not yet been reported. In the present study, increased rates of relative expression of genes encoding production of nisin were determined for the skim milk stored at 15 ℃ and challenged cheeses. However, some decreased amounts of *nis* genes expression were observed in the skim milk stored at 4 ℃. This difference might be related to the favorable physiological conditions at 15 ℃ rather than 4 ℃. Under 15 ℃, the level of nisin gene expression was lower than or similar with the initial value in the skim milk samples challenged with *P. fluorescens* and *B. cepacia,* as unexpected considering that pathogens significantly decreased during storage. This results may suggest that reduction of *P. fluorescens* and *B. cepacia* was due to another factors (e.g. acid) rather than nisin. In the skim

milk contaminated with *L. monocytogenes*, similarly as previously observed (Martinez et al., 2015), relative expression of nisin genes increased during fermentation and the results corresponded with the decrease of viable cell counts of pathogens.

At last, we tested the impact of *L.lactis* LDTM6804 on the sensory qualities of cheeses by using electronic nose. To evaluate the influence of bioprotective culture on the organoleptic properties of the food product, various methods were performed including sensory analyses with panels or gas chromatography(Budde et al., 2003, Comi et al., 2016). Unlike other methods, rapid discrimination of a sample from another and determination of evolutionary change of a sample are possible with electronic nose(Antoce and Namolosanu, 2011). Discrimination was achieved by a principal component analysis (PCA) and a discriminant factorial analysis (DFA) to predict the impact of *L.lactis* LDTM6804. Two groups were well separated among the samples, day 0 and Test 3 groups especially representing flavor change by *E. coli*. The result from DFA indicated clear grouping of cheeses within the addition of *L.lactis* LDTM6804. Although the cheeses from Test 1 and 2 group with no addition of *L.lactis* LDTM6804 showed distinctive profile pattern from the control group, the *L.lactis* LDTM6804 added cheeses were grouped within the control cheese. This may suggest that *L.lactis* LDTM6804 prevent shift of flavor patterns

caused by pathogenic bacteria.

In conclusion, this study highlights antimicrobial activity of *L.lactis* LDTM6804 as a potential bioprotective culture to regulate psychrotrophic spoilage and pathogenic bacteria in dairy products, especially showing broad spectrum of indicators and potential application in skim milk and cheese. Furthermore, flavor analysis data indicates that inhibition of spoilage and pathogenic bacteria by *L.lactis* LDTM6804 prevent undesirable flavor change compared to control cheese sample.

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Abstract in Korean

유가공 산업에서 우유 및 유제품의 미생물 부패는 품질과 안전성 측면에 있어 소비자들에게 중요한 문제로 여겨지고 있다. 더 안전한 유제품에 대한 소비자들의 요구로 인해 유산균의 생물보존제(biopreservative)로서의 이용이 증대되고 있는데, 유산균은 다양한 항균물질 (유기산, 과산화수소, 박테리오신 등)을 분비하면서 우유 및 유제품 내의 문제가 되는 부패성 및 병원성 미생물들을 억제한다. 따라서, 본 연구에서는 원유로부터 분리한 Lactococcus 속 유산균의 생물제어 종균으로서의 항균력을 유제품 모델에서 검증하고자 했다.

내냉성 부패 및 병원성 미생물에 대해 높은 항균력을 보이는 *Lactococcus lactis* subsp. *lactis* LDTM6804 (*L. lactis* LDTM6804) 균주가 원유로부터 분리되어 선발되었고, 이후 선발된 *L. lactis* LDTM6804 균주의 여러 가지 특성을 확인하였으며 유전체 분석을 실시하였다. *L. lactis* LDTM6804의 염색체 내에서 nisin Z 생합성과 관련한 유전자 클러스터가 발견되었으며 이 균주의 보존효과는 10 % 탈지유와 치즈 모델에서 확인하였다. *L. lactis* LDTM6804 균주를 탈지유에 6 log CFU/ml 수준으로 첨가하여 15 ℃ 에서 14일 배양하였을 때 *Pseudomonas fluorescens, Burkholderia cepacia, Listeria monocytogenes* 균이 각각 2.06, 1.29, 0.59 log 수준으로 *L. lactis* IL1403 균주가 첨가되었을때에 비교하여 감소하였다. 치즈 모델은 5 log CFU/g 수준의 *L. monocytogenes, Staphylococcus aureus, Escherichia coli* 균과 함께 접종되어 생산되었고 *L. lactis* LDTM6804균이 1 % 수준으로 기존의 발효종균과 함께 보조 접종되었다. 25일 숙성 이후 각각 0.42, 0.57, 0.74 log 수준이 *L. lactis* LDTM6804균주가 없는 대조군에 비하여 감소하였다. 탈지유와 치즈 모델의 보관기간 동안 quantitative real time PCR (qRT-PCR)

을 이용하여 nisin의 활성을 확인하였다. 마지막으로, 전자코기기를 이용하여 *L. lactis* LDTM6804 첨가에 의한 풍미변화를 분석하였다. 본 연구 결과에서 *L. lactis* LDTM6804균주는 내냉성 부패 및 병원성 미생물에 대해 강력한 항균력을 보였으며 풍미물질변화를 가져오지 않았다. 앞으로 적정 비율과 *L. lactis* LDTM6804균주의 발효물 및 정제 nisin 의 항균력에 관한 추가적인 연구가 필요할 것으로 보인다.