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ARTICLE

Physiological Characteristics and Production of Vitamin K_2 by *Lactobacillus fermentum* LC272 Isolated from Raw Milk

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Abstract

In order to develop a new starter culture for fermented milk, *Lactobacillus fermentum* LC272 was isolated from raw milk and its physiological characteristics were investigated. The vitamin K_2 concentration of *L. fermentum* LC272 was 184.94 μg/L in Rogosa medium and 63.93 μg/L in the reconstituted skim milk. The optimum growth temperature for *L. fermentum* LC272 was determined to be 40°C and it took 24 h for the pH to reach 5.2 under this condition. *L. fermentum* LC272 was more sensitive to rifampicin relative of the other 15 different antibiotics tested, and showed most resistance to streptomycin. *L. fermentum* LC272 showed higher activities to leucine arylamidase and acid phosphatase. It was comparatively tolerant to bile juice and acid and displayed high resistance against *Salmonella* Typhimurium and *Staphylococcus aureus* with rates of 82.9 and 86.3% respectively. These results demonstrated that *L. fermentum* LC272 could be an excellent starter culture for fermented milk with high levels of vitamin K_2 production.

Key words: *Lactobacillus fermentum*, vitamin K_2, physiological characteristics, fermented milk

Introduction

Vitamin K, which is a nutritional factor, plays a role in the regulation of bone metabolism. Vitamin K exists naturally in 2 forms, namely, vitamin K_1 (phylloquinone) in green plants and vitamin K_2 (menaquinone) in animals and bacteria. Vitamin K_1 is a single compound, but vitamin K_2 is a series of vitamins with multiisoprene units (one to four) at the 3-position of the naphtoquinone. Vitamin K_1 has an effect on bone metabolism (Hauschka *et al.,* 1975; Price, 1985). Vitamin K_2 is essential for the γ-carboxylation of osteocalcin, a bone matrix protein containing γ-carboxyglutamic acid which is synthesized in osteoblast of bone tissues (Hart *et al.,* 1985; Hauschka and Carr, 1982; Knapen *et al.,* 1989). Vitamin K is an essential cofactor for the formation of γ-carboxyglutamic acid (Gla) residues in proteins (Shearer, 1990). The Gla-containing proteins bind calcium ions and influence, for example, blood coagulation and tissue calcification (e.g., osteocalcin found in bone tissue) (Hauschka and Reid, 1978; Price *et al.,* 1976). Vitamin K deficiency has been implicated in several clinical ailments such as intracranial hemorrhage in newborn infants (Purves, 2005) and possible bone fracture resulting from osteoporosis (Iwamoto *et al.,* 2004). Lactic acid bacteria have been used as starter cultures to manufacture various foods and can be generally recognized as safe (GRAS), and a qualitative study has shown that some lactic acid bacteria produce menaquinone. Tani and Taguchi (1989) have reported that as much as 182 mg/L MK was produced using detergent supplement culture and a mutant of Flavobacterium. On the other hand, lactic acid bacteria were reported to produce menaquinones with the yield of 29-123 μg/L MK-7, MK-8, MK-9 and MK-10 (Morishita *et al.,* 1999). In many countries, the daily requirement for vitamin K is around 1 μg/kg of body weight (Collins and Jones, 1981).

The objective of this study is to develop a new starter for fermented milk and to investigate physiological characteristics.

Materials and Methods

Isolation of lactic acid bacteria

Raw milk samples (n=820) were collected from farms under the support of Seoul Dairy Cooperative and Provincial institute for livestock promotion in Korea. Strain LC272 was isolated from raw milk in modified MRS medium (Table 1). The strain was incubated in lactoba-

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Table 1. Composition of modified MRS agar

Component	g/L
Proteose peptone #3	10.0
Beef extract	10.0
Yeast extract	5.0
Lactose	20.0
Tween 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Sodium azide	0.25
Bromocresol purple	0.04
Agar	15

Table 2. Composition of modified Rogosa medium

Component	g/L
Dextrose	20.0
Trypticase peptone	20.0
Tryptose peptone	3.0
Yeast extract	5.0
Tri-ammonium citrate	2.0
K_2PO_4	3.0
K_2HPO_4	3.0
$MgSO_4 \cdot 7H_2O$	0.575
$FeSO_4 \cdot 7H_2O$	0.034
$MnSO_4 \cdot 4H_2O$	0.1
L-Cysteine · HCl	0.2
Sodium acetate	1.0
Tween 80	1.0

Table 3. Composition of reconstituted skim milk

Component	g/L
Skim milk powder	100.0
Yeast extract	5.0
$CaCl_2$	2.0
L-Cysteine · HCl	0.3

cilli MRS broth as the growth medium at 37°C for 24 h.

Identification of strain LC272

The properties of the strain LC272 was investigated by testing the Gram staining and microscopic observation after cultivation on tryptic soy agar for 24 h at 37°C. Bergey's Manual of Systematic Bacteriology was used to examine the morphological and physiological properties of the isolated strains. LC272 strain was identified by using the 16S rDNA sequencing method. Chromosomal DNA of isolated strain was separated by using SolGent Genomic DNA prep kit (SolGent, Korea).

The DNA extracts were used for polymerase chain reaction(PCR) with the universal primers (27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3')).

PCR were carried out in a programmable thermal cycler (PCR9700, ABI, USA; SolGent EF-Taq DNA polymerase, Korea), with the following steps : one cycle of denaturation at 95°C for 15 min, 30 cycles of 95°C for 20 s, 50°C for 40 s and 72°C for 90 s were performed. Final extension was carried out at 72°C for 5 min.

PCR product purified by using SolGent PCR purification kit(SolGent, Korea) was used for sequencing with a ABI 3730XL DNA analyzer (Applied Biosystems, USA).

Sample preparation

All organisms (10^7 CFU/mL) in Rogosa medium (or MRS medium) were inoculated into modified Rogosa medium (Table 2, Morishita *et al*., 1999) or reconstituted skim milk (Table 3, Morishita *et al*., 1999) and incubated at 35°C for 48 h. Medium was incubated 3 days at 37°C in the anaerobic condition with gas pack. Cultures (ca. 3×10^8 to 1×10^9 CFU/mL) were chilled in ice water to terminate growth. Cells were harvested from the MRS medium by centrifugation at 4,000 rpm for 15 min and were washed twice with saline and lyophilized. The fermented, reconstituted skim milk or Rogosa medium were lyophilized without harvesting cells, as described by Morishita *et al.* (1999).

Extraction of vitamin K

Extraction of vitamine K was carried out as described by Tsukamoto *et al.* (2001). One gram of sample lyophilized was added to 10 mL of deionized water and filtered after being mixed well. Then 5 mL of this filtered solution was added to the same volume of 2-propanol and mixed well for 15 min with shaking. 6 mL of hexane was added and mixed well for 15 min with shaking. 4 mL of the upper phase of the previous solution was evaporated under the stream of nitrogen and redissolved by adding 0.1 mL methanol. This solution was used for UPLC-Q-TOF MS after filtration by a cellulose acetate filter (0.45 μm). The standard vitamin K_2 (menaquinone-4, Sigma Chemical, St Louis, MO, USA) was dissolved in the methanol and used for the preparation of standard curve.

Analysis of vitamin K_2 by UPLC-Q-TOF MS

Vitamin K_2 was determined with an ultra performance liquid chromatography-quadrupole-time of flight (UPLC-Q-TOF). 8 μL sample was injected into the UPLC-Q-TOF MS (Waters, Milford, MA). The sample was injected

into an Acquity UPLC BEH C_{18} column (2.1×100 mm, 1.7 μm; Waters) in line with the UPLC system and equilibrated with methanol. Samples were eluted by methanol at a flow rate of 0.3 mL/min for 5 min. Absorbance of separated eluent was checked at 254 nm with photodiode array (PDA) detector and continue to measure the mass spectrum with Q-TOF. The Q-TOF was operated in ESI-negative mode with the capillary and sample cone voltage of 2.5 and 15 KV, source and desolvation temperature of 110°C, 300°C, desolvation and cone speed of 700 L/h and 20 L/h, respectively. Collision cell energy of precursor was 2 V but sample was raised up to 10-30 V. 200 pmole leucine was used as a lack mass. The 1st stage identification was done by elemental composition followed by the 2nd stage identification using online database (www.chemsider.com) and final determination and analysis was done using standard materials.

Growth of strain

The number of viable *L. fermentum* LC272 was determined by serial 10-fold dilution in 0.1% peptone water. *L. fermentum* LC272 was inoculated 50 μL (9.6×10^5 CFU/mL) into 150 mL of 10% reconstituted skim milk. And then culture was incubated to 3 h interval until 24 h at 34°C, 37°C and 40°C. All pour plates were incubated aerobically at 37°C for 48 h using BCP plate count agar.

Antibiotic tolerance

L. fermentum LC272 was grown at 37°C for 18 h on MRS broth. Minimal inhibitory concentration (MIC) was determined when its strain did not grow on Tryptic soy broth (Difco, USA) with each concentration of antibiotics after incubation at 37°C for 48 h.

Enzyme activity

The API ZYM kit (BioMerieux, Lyon, France) was used to study enzyme activity. *L. fermentum* LC272 was grown at 37°C for 18 h on MRS broth. Sediment from centrifuged broth culture was used to prepare the suspension at 10^5-10^6 CFU/mL. After inoculation, cultures were incubated for 5 h at 37°C. Placing a surface active agent (ZYM A reagent) in the cupules facilitated solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and values from 0-5 corresponding to the colors developed, were assigned. The approximate number of free nmol hydrolyzed substrate was determined based on the color strength: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 or higher.

Bile tolerance

Bile tolerance was carried out as described by Gilliland and Walker (1990). *L. fermentum* LC272 was grown at 37°C for 18 h on the MRS broth. Culture of *L. fermentum* LC272 was compared for their ability to grow in the presence of bile by individual inoculation (1%) into sterile MRS broth containing 0.05% L-cysteine with and without 0.3% oxgall. After plating for initial counts, mixtures were incubated anaerobically for 7 h at 37°C. *L. fermentum* LC272 was then enumerated again to test for survival rates after 7 h incubation. All pour plates were incubated anaerobically for 48 h at 37°C.

pH tolerance

pH tolerance was carried out as described by Clark *et al*. (1993). Solutions of 37% HCl in double-distilled water were adjusted to pH level to 2.0, 3.0, and 4.0. Sterile double-distilled water (pH 6.4) served as the control. 10 mL of each pH solution were transferred into sterile test tubes.

1 mL of stock culture containing approximately 10^9 CFU/mL of *L. fermentum* LC272 using MRS agar containing 0.05% cysteine was then transferred into each of the four pH solutions. The pH solutions containing *L. fermentum* LC272 were then incubated anaerobically at 37°C, followed by intermittent plating after 1, 2, and 3 h to stimulate survival of *L. fermentum* LC272 under pH conditions common to the human stomach. Samples from the pH solution were taken at 1, 2 and 3 h after the samples was resuspended and subjected to serial dilutions. About 100 uL above sample solution was spread onto the surface of BCP plate count agar plates and incubated anaerobically at 37°C for 48 h.

Antimicrobial activity

Antimicrobial activity was carried out as described by Gilliland and Speck (1977). *Escherichia coli* KFRI 242, *Salmonella* Typhimurium KFRI 251, *Staphylococcus aureus* KFRI 219 were from the culture collection of the Korea Food Research Institute. *Escherichia coli* was enumerated on EMB agar, *Salmonella* Typhimurium on Bismuth sulfite agar, and *Staphylococcus aureus* on Baird parker agar. All plate were incubated 48 h at 37°C.

The control and associative culture were incubated for 6 h in a water bath at 37°C. At the end of the incubation time, samples were removed and placed in an ice bath until analyzed. The number of CFU of pathogens per mL was determined using the appropriate selective medium and in some experiments the pH of the samples was also

measured. Percentages of inhibition were determined using the following formula :

$$\text{Inhibition (\%)} = \frac{\text{(CFU/mL in control)} - \text{(CFU/mL in associative culture)}}{\text{(CFU/mL in control)}} \times 100$$

Results and Discussion

Isolation of lactic acid bacteria

Total 820 raw milk samples were collected from farms under the support of Seoul Dairy Cooperative and Provincial institute for livestock promotion in Korea. Total 1505 strains were isolated as a lactic acid bacteria from raw milk in modified MRS medium (Table 1).

Selection of lactic acid bacteria producing vitamin K_2

Four strains of M581, M301, M351, LC272 were selected as a high vitamin K_2-producing lactic acid bacteria, which is more than 30 μg/L in Rogosa medium and 20 μg/L in reconstituted skim milk. Fig. 1 shows chromatogram analysised by UPLC-Q-TOF MS. The concentration of vitamin K_2 was determined by the calculation of peak area with the retention time of 2.29 minutes same as that of standard sample.

Table 4 shows the vitamin K_2 (menaquinone-4) concentration of 4 separated strains with UPLC-Q-TOF MS method and the highest was 184.94 μg/L in Rogosa medium and 63.93 μg/L in the reconstituted skim milk. Vitamin K_2 production of LC272 strain in Rogosa medium showed 3 times higher than that in reconstituted skim milk while other strains showed 1.5-5 times. These results might explain that the media formula and kinds of lactic bacteria may result in the changes of vitamin K_2 synthesis.

Compared to the result of Morishita *et al.* (1999), vitamin K_2 (MK-7) production of 2 strains of *Lactococcus lactis* ssp. *cremoris* was 90 μg/L and 29 μg/L, respectively, and it is comparable level to the LC272. Hojo *et al.* (2007) has reported that the menaquinone-4 concentration in the Gruyere cheeses were 81 and 96 ng/g, whereas in the Appenzeller cheeses were 43 and 52 ng/g, respectively. To confirm the vitamin K_2 production by the selected LC 272, product ion of the LC 272, 444.3003 m/z was confirmed using LC MS/MS system. Compared to the results of Table 5, it was possible to confirm that the LC 272 produced material that belongs to the 444 Da of nominal mass and finally it is vitamin K_2.

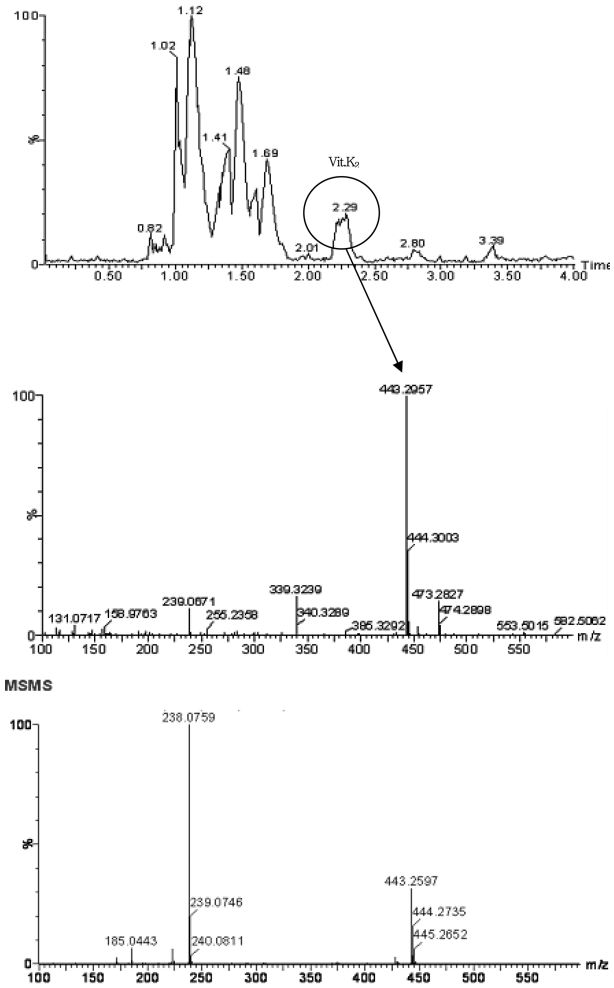

Fig. 1. Chromatogram of Vitamin K_2 by LC/Mass Spectrometry.

Table 4. Vitamin K_2 content of Rogosa medium and reconstituted skim milk produced by selected lactic acid bacteria (μg/L)

Strains	Rogosa medium	Reconstituted skim milk
M581	36.12±4.53	20.25±8.64
M301	38.54±5.14	27.65±4.33
M351	155.58±11.72	30.93±5.25
LC272	184.94±15.51	63.93±9.28

$R^2 = 0.9845$, n=3

Table 5. Inherent properties of standard vitamin K_2 (menaquinone-4)

Parameters	Inherent properties
Empirical formula	$C_{31}H_{40}O_2$
Molecular weight	444.6481 Da
Normal mass	444.0000 Da
Monoisotopic mass	444.3028 Da
Systematic name	2-methyl-3-[(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl]naphthalene-1,4-dione

Identification and DNA sequencing of selected strain LC272

It was examined the physiological and biochemical test to determine genus and species of selected LC272 strain. Selected LC272 strain was non-spore, rod type, hetero fermentative, gram positive bacteria and exhibited negative properties on catalase and motility. Also, it can not grow at 15°C but it can grow 45°C. It does not produce gas and ammonia from glucose and arginine so that it was identified as a genus *Lactobacillus* (Table 6).

Identification using the 16S rDNA sequencing method by the PCR of universal primer was result in the *Lactobacillus fermentum* with possibility of 99% (data not shown). Based upon the result of previous study, it has named as a *Lactobacillus fermentum* LC272.

Table 6. Physiological characteristics of *L. fermentum* LC272

Gram reaction		+	
Cell type		rod	
Spore forming		-	
Motility		-	
Aerobic growth		+	
Anaerobic growth		+	
Catalase reaction		-	
Growth at 15°C		-	
Growth at 45°C		+	
Gas forming from glucose		-	
Ammonia production from alginin		-	
Acid production from			
Glycerol	-	Salicin	-
Erythritol	-	Cellobiose	-
D-Arabinose	-	Maltose	+
L-Arabinose	-	Lactose	+
Ribose	+	Melibiose	+
D-Xylose	-	Saccharose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inulin	-
β-Methyl-D-xyloside	-	Melezitose	-
Galactose	+	D-Raffinose	+
D-glucose	+	Starch	-
D-Fructose	+	Glycogen	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	-
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	+	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-Acetyl glucosamine	-	Gluconate	+
Amygdalin	-	2-Keto-gluconate	-
Arbutin	-	5-Keto-gluconate	-
Esculin	-		

Growth of strain

L. fermentum LC272 was incubated at 34°C, 37°C and 40°C for 24 h by checking 3 h term. Log phase time was until 6 h incubation and generation time was 0.60 h at 34°C, 0.58 h at 37°C and 0.52 h at 40°C, respectively. Finally, the optimum growth temperature of *L. fermentum* LC272 was 40°C and it has taken 24 h to reach the pH to 5.2 under this condition (Fig. 2).

Antibiotic tolerance

It is very important for the probiotic strain can survive in the antibiotic circumstance. Table 7 shows the toler-

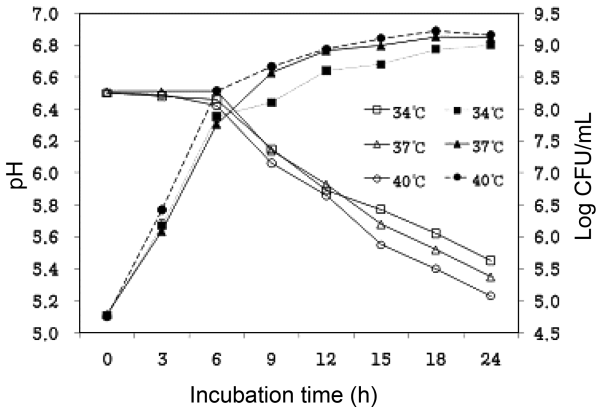

Fig. 2. Growth and pH changes of *L. fermentum* LC272 in 10% reconstituted skim milk at various temperatures.

Table 7. Antibiotic susceptibility of *L. fermentum* LC272

Antimicrobial Agents	Minimal inhibitory concentrations (μg/mL)
Aminoglycosides	
Amikacin	200
Gentamycin	1600
Kanamycin	10
Neomycin	120
Streptomycin*	2400
β-Lactams	
Penicillin-G*	0.5
Methicillin	60
Oxacillin	400
Ampicillin	25
Gram-positive spectrum	
Bacitracin*	160
Rifampicin	0.1
Novobiocin	320
Lincomycin	40
Gram-negative spectrum	
Polymyxin B*	160
Broad spectrum	
Chloramphenicol	2.5
Vancomycin	7.8

*units/mL, n=3

ance of *L. fermentum* LC272 strain on the 16 kinds of antibiotics. *L. fermentum* LC272 showed more sensitive to rifampicin and penicillin-G in a comparison of 14 different antibiotics, and showed most resistance to streptomycin and gentamycin.

Lim *et al.* (2010) has reported that *L. paracasei* subsp. *paracasei* BFI46 was sensitive to penicillin-G and chloramphenicol but it shown high resistance to neomycin, kanamycin and polymycin.

Enzyme activity

Table 8 shows the enzyme activity of *L. fermentum* LC272. The leucine arylamidase and acid phosphatase activity was 5 and it was relatively high on the other hand β-glucuronidase activity one of carcinogen-producing enzymes in the gut was 0 and it was no enzyme activity. This result is similar trends to the report of Lim *et al.* (2010) on the β-glucuronidase activity of *L. paracasei* subsp. *paracasei* BFI46.

Bile tolerance

Fig. 3 shows growth curves in MRS broth or MRS broth containing 0.3% bile. The log value of population after 7 hours incubation without 0.3% oxgall was 8.9 but 8.1 with the addition of 0.3% bile so that it has shown good bile tolerance. This result is similar trends to the report of Lim *et al*. (2010) on the bile tolerance of *L. paracasei* subsp. *paracasei* BFI46 that it has shown slight decrease in the log value of population from 9 to 8.5. Bile salts are toxic effect for living cells, since they disorganize the structure of the cell membrane and bile salt tolerance is considered one of the essential properties required for lactic acid bacteria to survive in the small intestine (Succi *et al*., 2005).

Table 8. Enzyme patterns of *L. fermentum* LC272*

Enzyme	*Lactobacillus fermentum* LC272
Alkaline phosphatase	1
Esterase (C4)	4
Esterase lipase (C8)	4
Lipase (C14)	0
Leucine arylamidase	5
Valine arylamidase	2
Cystine arylamidase	2
Trypsin	1
Chymotrypsin	1
Acid phosphatase	5
Naphthol-AS-BI-phosphohydrolase	4
α-Galactosidase	0
β-Galactosidase	2
β-Glucuronidase	0
α-Glucosidase	0
β-Glucosidase	0
N-Acetyl-β-glucosaminidase	0
α-Mannosidase	0
α-Fucosidase	0

*A value ranging from 0 to 5 is assigned to the standard color, Zero represents a negative; 5 represent a reaction of maximum intensity. Values 1 through 4 represent intermediate reactions depending on the level of intensity. The approximate activity may be estimated from the color strength; 1 corresponds to the liberation of 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles and 5 to 40 nanomoles or more (n=3).

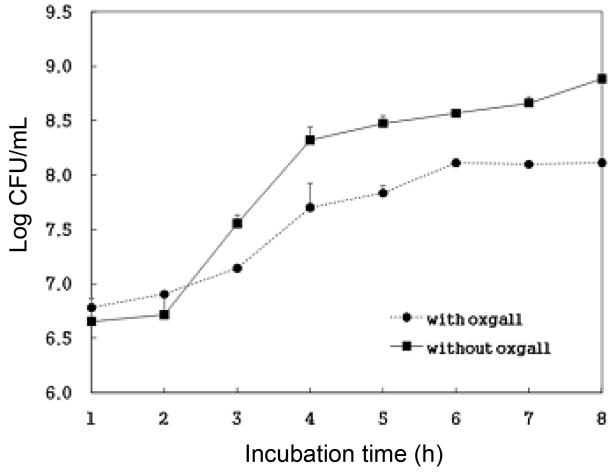

Fig. 3. Growth curve of *L. fermentum* LC272 in MRS broth containing 0.05% L-cysteine with and without 0.3% oxgall.

Acid tolerance

Acid tolerance is a property that any stain is expected to have effects in GI tract should possess (Maragkoudakis *et al*., 2006). To be a good probiotic, it is necessary to survive in the pH lower than 3 so that it could reach to the

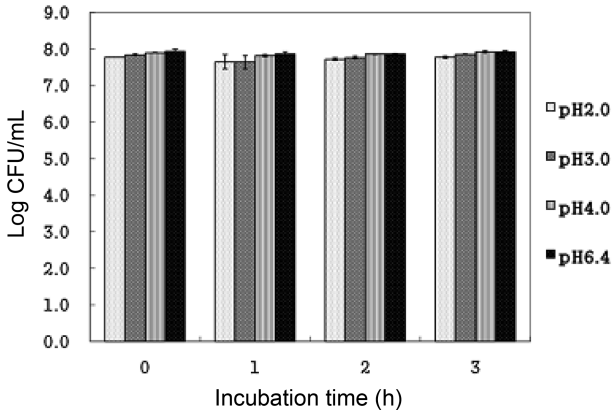

Fig. 4. Survival of *L. fermentum* LC272 after 3 h in HCl solution (pH 2.0, 3.0, 4.0, and 6.4).

Table 9. Growth inhibition of pathogens by *L. fermentum* LC272 in the MRS broth

Pathogens	Pathogens[a]		*L. fermentum* LC272[a] + Pathogens		Inhibition (%)
	CFU/mL	pH	CFU/mL	pH	
Escherichia coli	$2.8\times10^7\pm4.2\times10^6$	6.65	$5.3\times10^8\pm2.1\times10^7$	4.81	0.0
Salmonella Typhimurium	$7.0\times10^6\pm1.4\times10^5$	6.48	$1.2\times10^6\pm4.6\times10^4$	4.75	82.9
Staphyloccous aureus	$1.6\times10^8\pm1.8\times10^6$	6.53	$2.2\times10^7\pm5.0\times10^5$	4.83	86.3

*Initial count *Lactobacillus fermentum* LC272 : $1.5\times10^6\pm8.2\times10^4$ CFU/mL (n=3)
[a]Determined after 6 h of incubation at 37°C

small intestine through the stomach (Booth, 1985; Mcdonald *et al.*, 1990). The acid tolerance of lactic acid bacteria has been linked to the induction of H^+-ATPase activity (Matsumoto *et al*., 2004; Ventura *et al*., 2004). Therefore, the variation in the acid tolerance of the selected probiotics might be related to the difference in H^+-ATPase activity in the probiotics.

Fig. 4 shows the pH tolerance of *L. fermentum* LC272. As the test pH was increased to pH 2, 3, 4 and pH 6, it has shown no change. *L. fermentum* LC272 is not influenced by low pH. And this result was similar to the report of Lim *et al*. (2010) on the *L. paracasei* subsp. *paracasei* BFI46.

Antimicrobial activity

The antimicrobial ability is one of important property for probiotics. The antimicrobial activity of lactic acid bacteria may be due to a number of factors. Among these there are factors including decreased pH levels, competition for substances with a bactericidal or bacteriostic action, including bacteriocins (Parente and Ricciardi, 1999). Table 9 shows the antimicrobial activity of *L. fermentum* LC272 against the pathogenic strains. *L. fermentum* LC272 showed high inhibition rate against *Salmonella* Typhimurium and *Staphylococcus aureus* with the rate of 82.9 and 86.3%, respectively. But it has not shown antimicrobial ability against *Escherichia coli.* The pH of media of pathogenic strain was 6.48-6.65 on the other hand, the pH of mixed strain media of pathogenic strain and *L. fermentum* LC272 was 4.75-4.83 due to the acid production of *L. fermentum* LC272. Lim *et al*. (2010) has reported that *L. paracasei* subsp. *paracasei* BFI46 has antimicrobial activity on the *E. coli, S.* Typhimurium and *S. aureus* with the rate of 57.14%, 96.36% and 0%, respectively. *L. fermentum* LC272 showed higher antimicrobial activity against *S. aureus.*

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References

1. Booth, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49**, 359-378.
2. Clark, P. A., Cotton, L. N., and Martin, J. H. (1993) Selection of bifidobacteria for use as dietary adjuncts in cultured dairy foods: II-Tolerance to simulated pH of Human Stomachs. *Cultured Dairy Products J.* **28**, 11-14.
3. Collins, M. D. and Jones, D. (1981) Distribution of isoprenoid quinine structural types in bacteria and their taxonomic implication. *Microbiol. Rev.* **45**, 316-354.
4. Gilliland, S. E. and Speck, M. L. (1977) Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. *J. Food Prot*. **40**, 820-823.
5. Gilliland, S. E. and Walker, D. K. (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J. Dairy Sci.* **73**, 905-911.
6. Hart, J. P., Sheaver, M. J., Klenerman, L., Catterall, A., Reeve, J., Sambrook, P. N., Dodds, R., A., Bitenky, and Chayen, J. (1985) Electrochemical detection of depressed circulating levels of vitamin K_1 in osteoporosis. *J. Clin. Endocrinol. Metab.* **60**, 1268-1269.
7. Hauschka, P. V., Lian, J. B., and Gallop, P. M. (1975) Direct identification of the calcium binding amino acid, γ-carboxyglutamate, in mineralized tissues. *Proc. Natl. Acad. Sci. USA* **72**, 3925-3929.
8. Hauschka, P. V. and Carr, S. A. (1982) Calcium-dependent α-helical structure in osteocalcin. *Biochem.* **21**, 2538-2547.
9. Hauschka, P. V. and Reid, M. L. (1978) Vitamin K dependence of a calcium-binding protein containing gamma-carboxyglutamic acid in chicken bone. *J. Biol. Chem.* **253**, 9063-9068.
10. Hojo, K., Watanabe, R., Mori, T., and Taketomo, N. (2007) Quantitative measurement of tetrahydromenaquinone-9 in cheese fermented by propionibacteria. *J. Dairy Sci.* **90**, 4078-4083.
11. Iwamoto, J., Takeda, T., and Sato, Y. (2004) Effects of vitamin K_2 on osteoporosis. *Curr. Pharm. Des.* **10**, 2557-2576.
12. Knapen, M. H. J., Hamulyak, K., and Vermeer, C. (1989) The effect of vitamin K supplementation on circulating osteocalcin (bone gla protein) and urinary calcium excretion.

Ann. Intern. Med. **111**, 1001-1005.
13. Lim, S. D., Kim, K. S., Cho, S. A., and Do, J. R. (2010) Physiological Characteristics and Immunomodulating Activity by *Lactobacillus paracasei* subsp. *paracasei* BFI46 Isolated from New-Born Infant Feces. *Korean J. Food Sci. Ani. Resour*. **30**, 223-231.
14. Matsumoto, M., Ohishi, H., and Benno, Y. (2004) H^+-ATPase activity in bifidobacterium with special reference to acid tolerance. *Int. J. Food Microbiol.* **93**, 109-113.
15. Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., and Tsakalidou, T. (2006) Probiotic potential of Lactobacillus strains isolated from dairy products. *Int. Dairy J*. **16**, 189-199.
16. Mcdonald, L. C., Fleming, H. P., and Hassan, H. M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus casei. Appl. Environ. Microbiol.* **53**, 2124-2128.
17. Morishita, T., Tamura, N., Makino, T., and Kudo, S. (1999) Production of manaquinones by lactic acid bacteria. *J. Dairy Sci*. **82**, 1897-1903.
18. Parente, E. and Ricciardi, A. (1999) Production, recovery and purifications of bacteriocins from lactic acid bacteria. *Appl. Microbiol. Biotechnol.* **52**, 628-638.
19. Price, P. A. (1985) Vitamin K-dependent formation of bone gla protein (osteocalcin) and its function. *Vitam. Horm.,* **42**, 65-108.
20. Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J., and Raman, N. (1976) Characterization of a gamma-carboxyglutamic acid-containing protein from bone. *Proc. Natl. Acad. Sci. USA* **73**, 1447-1451.
21. Purves, E. (2005) Neonatal hematologic disorders. *J. Pediatr. Oncol. Nurs.* **22**, 168-175.
22. Shearer, M. J. (1990) Vitamin K and vitamin K-dependent proteins. *Br. J. Haematol.* **75**, 156-162.
23. Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., and Pacifico, S. (2005) Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *FEMS Microbiol. Lett*. **244**, 129-137.
24. Tani, T. and Taguchi, H. (1989) Extracellular production of menaquinone-4 by mutant of Flavobacterium sp. 238-7 with a detergent supplemented culture. *J. Ferment. Bioeng.* **67,** 102-106.
25. Tsukamoto, Y., Kasai, M., and Kakuda, H. (2001) Construction of a Bacillus subtilis (natto) with high productivity of vitamin K_2 (Menaquinone-7) by analog resistance. *Biosci. Biotechnol. Biochem*. **65**, 2007-2015.
26. Ventura, M., Canchaya, C., van Sinderen, D., Fitzgerald, G. F., and Zink, R. (2004) *Bifidobacterium lactis* DSM 10140: identification of the atp (atpBEFHAGDC) operon and analysis of its genetic structure, characteristics, and phylogeny. *Appl. Environ. Microbiol.* **70**, 3110-3121.

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