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ARTICLE

Physiological Characteristics and Immunomodulating Activity of *Streptococcus macedonicus* LC743 Isolated from Raw Milk

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Abstract

To develop a new starter culture for fermented milk, *Streptococcus macedonicus* LC743 was isolated from raw milk and its physiological characteristics were investigated. *S. macedonicus* LC743 showed good immunomodulating activity compared to the index LAB starters tested. The optimum growth temperature of *S. macedonicus* LC743 was 40°C, and it took 18 h to reach pH 4.34 under these conditions. *S. macedonicus* LC743 showed higher sensitivity to novobiocin in a comparison of 15 different antibiotics and showed the highest resistance to gentamycin. It also showed higher activities of leucine arylamidase and acid phosphatase. Moreover, it was comparatively tolerant to bile juice and acid and displayed high resistance to *Escherichia coli, Salmonella Typhimurium*, *Staphylococcus aureus* with rates of 80.0%, 68.42%, and 81.54% respectively. These results demonstrate that *S. macedonicus* LC743 could be an excellent starter culture for fermented milk with a high level of immunomodulating activity.

Key words: *Streptococcus macedonicus*, immunomodulating activity, physiological characteristics, fermented milk

Introduction

Especially lactic acid bacteria (LAB), that are part of the human commensal microbial and that have a long history of use in food products, have been studied widely. The ability of several LAB strains to modulate host innate as well as acquired immune responses, has been demonstrated in many *in vitro* experiments and animal models (Baken *et al*., 2006). Certain lactic acid bacteria, orally administered, induce the activation of peritoneal macrophages, which may be important effecter cells in specific and nonspecific host defense (Perdigon *et al.*, 1986).

Interactions of LAB and their products with immunocompetent cells such as macrophages and T-cells can lead to the production of cytokines which have a manifold effect on immune and non-immune cells (Nussler and Thomson, 1992). Cytokines play a key role as communication signals during both normal immunologic responses and pathologic conditions leading to infectious, inflammatory, and neoplastic diseases. Production and biologic function of cytokines are affected by changes in macro- and micronutrients (Meydani, 1990). LAB could induce the production of cytokines through two possible pathways. Cytokine secretion can be released after antigen presentation to T-lymphocytes. or cytokine production results from the direct interaction between LAB and immunocompetent cells (Taguchi *et al*., 1991; Lefrancois, 1994). The presence of specific receptors for peptidoglycan a compound of the LAB cell wall was manifested on lymphocytes and macrophages (Dziarski, 1991). The ability of peptidoglycan to induce the secretion of Interleukin 1 (IL-1), Interleukin 6 (IL-6) and Tumor necrosis factor α (TNF-α) by monocytes was proved by Bhakdi *et al*. (1991), Tufano *et al*. (1991), Heumann *et al*. (1994). Cytokines influence the defence system of the host directly or indirectly. Soluble factors such as IL-1, IL-6 and TNF-α released by monocytes have been shown to play key roles in proliferation, activation and differentiation of immune cells (Czarniecki, 1993). Also, IL-1 stimulates T-and B-cell proliferation, TNF-α has a cytotoxic effect on tumour cells (Gill, 1998). IL-1 is a major product of the stimulated monocyte and is responsible for diverse biological effects. The systemic effects of IL-1 include fever, increased circulating neutrophils, hepatic acute phase proteins, slow wave sleep, elevated insulin levels and hypotension (Dinarello, 1987). Nitric oxide

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(NO) is also an important molecule that regulates a wide range of biological activities in the nervous, immune, and vascular systems, and it is implicated in a number of different pathologies both in animals and humans (Boje, 2004; Brennan and Moncada, 2002; Chen *et al.,* 2003; Guzik *et al.,* 2003; Moncada, 1999; Zeidler *et al.,* 2004; Zucker *et al.,* 2004).

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

820 kinds of raw milk samples were collected from farms under the support of Seoul Dairy Cooperative and Provincial institute for livestock promotion in Korea. Strain LC743 was isolated from raw milk in modified MRS medium (Table 1). The strain was incubated in lactobacilli MRS broth as the growth medium at 37°C for 18 h.

Identification of strain LC743

The properties of the strain LC743 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. LC743 strain were 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 (solgent EF-Taq, 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).

Table 1. Composition of modified MRS aga

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

Cell culture

RAW 264.7 macrophage cell was obtained from Korean Cell Line Bank (KCLB, Korea). Cell were maintained in DMEM including 10% of fetal bovine serum, penicillin-streptomycin of 100 units/mL at 5% in a CO_2 incubator and were collected and stored until its analysis for cytokine assay of tumor necrosis factor-α (TNF-α) and nitric oxide (NO).

Sample preparation

All organisms were inoculated in MRS broth, and then incubated at 37°C for 18 h (until entry about 10^9 CFU/mL). Cells were centrifugated at 1,500 g, for 15 min. The supernatant was removed and the pellet washed once with a physiological saline solution, and resuspended in 1.5 mL a Hanks' buffered salt solution, and then heated at 100°C for 50 min, as described by Marin *et al* (1997). Sample (10^8 CFU/mL or 10^9 CFU/mL) and RAW264.7 (2×10^5 cells/mL) were cultured in 96-well plate with and without *Escherichia coli* lipopolysaccharide (LPS, 1 μg/mL; Sigma) and then incubated at 37°C in the CO_2 incubator. After 48 h, supernatants were collected and used to analyze NO and cytokine assay (IL-1 α, TNF- α).

Nitric oxide production

Nitric oxide (NO) production was assessed by measuring nitrite accumulation, a stable metabolic product of nitric oxide, in the culture supernatants. Nitrite concentrations were determined by the Griess reaction. Briefly, equal amounts of naphthylethylene diamine dihydrochloride (100 mg dissolved in 100 mL of distill water) and sulfanilamide (1 g dissolved in 100 mL of a 2.5% phosphoric acid solution) solutions were mixed prior to each assay (Griess reagent or chromogenic reagent). Nitrite standards (2 mM stock solution; from 0 to 200 μM) were diluted in the same media in which the cells were suspended. Equal amounts of Griess reagent and $NaNO_2$

standards or samples (50 μL) were placed in a 96-well plate in duplicate and incubated for 10 min at room temperature to allow the chromophore to develop and stabilize. Absorbance was read at 540 nm using the ELISA reader (Molecular Device, USA).

Cytokine assay

The secretion of cytokines was determined by using interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) kits according to manufacture's instruction (Komabiotech; Korea). IL-1α was determined by using the following procedure; 100 μL of standards or samples was added to each well in duplicate. Plate was covered and incubated at room temperature for 2 h. The wells were aspirated to remove liquid and plate was washed 4 times. 100 μL of prepared detection antibody was added to each well. Plate was covered and incubated at room temperature for 2 h. Liquid was removed and plate was washed 4 times. 100 μL of prepared Streptavidin-HRP Solution was added to each well. Plate was covered and incubated at room temperature for 30 min. Liquid was removed and plate was washed 4 times. 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution was added to each well. Plate was incubated at room temperature for a proper color development. Reaction was stopped by adding 100 μL of 2 M sulfuric acid to each well. Absorbance was measured on a ELISA reader (Molecular device, USA) at 450 nm.

TNF-α was determined by using the following procedure; 100 μL of standards or samples was added to each well in duplicate. Plate was covered and incubated at room temperature for 2 h. The wells were aspirated to remove liquid and plate was washed 4 times. 100 μL of prepared detection antibody was added to each well. Plate was covered and incubated at room temperature for 2 h. Liquid was removed and plate was washed 4 times. 100 μL of prepared Streptavidin-HRP Solution was added to each well. Plate was covered and incubated at room temperature for 30 min. Liquid was removed and plate was washed 4 times. 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution was added to each well. Plate was incubated at room temperature for a proper color development. Reaction was stopped by adding 100 μL of 2 M sulfuric acid to each well. Absorbance was measured on a ELISA reader (Molecular device, USA) at 450 nm.

Growth of strain

The number of viable *S. macedonicus* LC743 was determined by serial 10-fold dilution in 0.1% peptone water. *S. macedonicus* LC743 was inoculated 50 μL (9.6×10^5/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

S. macedonicus LC743 was grown at 37°C for 18 h in MRS broth and inoculated (1%, v/v) in Tryptic soy broth (Difco, USA) supplemented with antibiotics (amikacin, gentamicin, kanamycin, neomycin, streptomycin, penicillin-G, methicillin, oxacillin, ampicillin, bacitracin, rifampicin, novobiocin, lincomycin, polymyxin B, and chloramphenico; Sigma) at various concentrations of 2-fold dilution step. Minimal inhibitory concentration (MIC) was determined by the checking the moment of the strain stop growing after incubation at 37°C for 48 h.

Enzyme activity

The API ZYM kit (bioMerieux, Lyon, France) was used to study enzyme activity. *S. macedonicus* LC743 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). *S. macedonicus* LC743 was grown at 37°C for 18 h on the MRS broth. Culture of *S. macedonicus* LC743 were 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. *S. macedonicus* LC743 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 *S. macedonicus* LC743 using MRS agar containing 0.05% cysteine was then transferred into each of the four pH solutions. The pH solutions containing *S. macedonicus* LC743 were then incubated anaerobically at 37°C, followed by intermittent plating after 1, 2, and 3 h to stimulate survival of *S. macedonicus* LC743 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 μL 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. *E. coli* was enumerated on EMB agar, *S. Typhimurium* on Bismuth sulfite agar, and *S. aureus* on Baird parker agar. All plate were incubated 48 h at 37°C.

The control and associative culture were incubated 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 and selection of lactic acid bacteria

820 kinds of raw milk samples were collected from farms under the support of Seoul Dairy Cooperative and Provincial Institute for Livestock Promotion in Korea. 1505 strains of lactic acid bacteria were isolated from the raw milk in the modified MRS medium (Table 1). Among them 512 strains were selected by the incubation at 37°C for 18 h in the 10% reconstituted skim milk and reach the pH to 4.5 and coagulated.

Selection of strain of immunomodulating activity

The strain coagulated skim milk by the incubation at 37°C for 18 h was selected for the immunomodulating activity. All strains were inoculated in MRS broth, and then incubated at 37°C for 12 h (until cell desity reach to 10^9 cells/mL) and then collected by centrifugation at 1,500×g, for 15 min. LC743 strain were selected by the succinylation of MRS broth and washed with a physiological saline solution, and resuspended in 1.5 mL a Hanks' buffered salt solution, and heated at 100°C for 50 min, as described by Marin *et al.* (1997). And then continued by the IL-1α and TNF-α test. IL-1α is involved in the regulation of immune responses, inflammation, and in the activation of host defense machinery. It is mainly produced by the activated macrophages and many other different types of cells including fibroblasts, T cells, B cells, astrocytes, and keratinocytes (Dinarello, 1991). TNF-α is also produced by activated macrophages, fibroblasts, and many different types of cells. The regulation of IL-1α and TNF-α production is critical in the maintenance of homeostasis of the immune system and in the prevention and treatment of the immune diseases (Kang *et al.*, 1996). A number of investigations suggest that lactic acid bacteria could immunopotentiate the gut mucosal immune system via activation of macrophages and production of cytokines (Perdigon *et al*., 1992). In addition to cytokines, leukocytes can produce reactive oxygen intermediates involved in immune response to invading microorganism. One of the most important mediators is nitric oxide (Tejada-Simon *et al*., 1999). Table 2 shows the comparison of immunomodulating activity of selected and index strains. The selected LC743 strain was proven to be an excellent immunomodulating activity by the high interlukin-1α secretion, NO secretion and TNF secretion activity. And this result was superior to the report of Lim *et al.* (2010) that *L. paracasei* subsp. *paracasei* BFI46 has immunomodulating activity on IL-1α, TNF-α and NO with the value of >1000 pg/mL, >2450 pg/mL and 19.58 μM, respectively. Fig. 1 shows the effect of LC743 on IL-1α, TNF-α and NO production in RAW 264.7 cells cultured with and without LPS (1 μg/mL). In both unstimulated and mitogen-stimulated cells, the effects on IL-1α, TNF-α and NO production were more pronounced with concentrations of LC743 increased from 10^8 CFU/mL to 10^9 CFU/mL. In the addition of LPS stimulation, the con-

Table 2. Effect of lactic acid bacteria on the production of IL-1α, TNF α, and NO in Raw 264.7 cells

Strains	Source	dose (CFU/mL)	IL-1α (pg/mL)	TNF-α (pg/mL)	NO (μM)
Lactobacillus acidophilus	ATCC 11506,	10^8	99.21±21.64	1594.19±21.67	ND[1)]
	IFO 3205	10^9	1080.33±24.30	2582.03±38.32	23.91±2.53
Lactobacillus casei	ATCC 393	10^8	112.53±25.94	1673.44±34.83	ND
		10^9	1080.9±20.54	2195.24±26.38	4.21±1.92
Lactobacillus lactis subsp. *lactis*	ATCC 21053	10^8	125.12±29.31	1374.28±35.26	ND
		10^9	1161.57±36.95	2100.98±29.01	13.04±1.01
Lactobacillus fermentum	ATCC 14931	10^8	68.56±15.42	1358.62±24.62	ND
		10^9	445.21±19.87	2219.42±81.57	6.52±1.42
Lactobacillus bulgaricus	ATCC 33409	10^8	92.37±17.34	1289.31±27.05	ND
		10^9	1110.94±21.25	2204.91±41.22	6.69±2.03
Lactococcus cremoris	KFRI 349	10^8	71.25± 9.54	1687.33±14.37	ND
		10^9	370.05±54.23	2599.03±27.54	18.40±3.40
Lactobacillus helveticus	ATCC 15009	10^8	168.13±15.42	1906.42±11.09	ND
		10^9	1912.53±62.34	2732.22±36.04	4.49±2.73
Lactobacillus casei	KFRI 709	10^8	59.24±11.80	2085.14±67.12	ND
		10^9	371.76±12.11	3302.04±19.21	21.73±1.84
Lactobacillus brevis	ATCC 8287,	10^8	80.15± 6.27	1973.28±44.70	ND
	IFO 3345	10^9	695.04±29.73	2995.12±72.63	13.04±1.77
Lactococcus lactis	NRRL B-633	10^8	51.92± 8.25	1925.7±40.16	ND
		10^9	580.59±60.01	3104.56±51.93	6.52±2.76
Lactococcus diacetylactis	NRRL B-2356	10^8	85.10± 9.14	1958.63±41.28	ND
		10^9	960.74±51.92	3021.24±38.26	4.34±2.99
Lactobacillus delbrueckii subsp.	ATCC 7830,	10^8	72.51±10.66	1694.57±19.07	ND
lactis	IFO 3376	10^9	693.01±21.28	2610.71±45.94	18.14±3.05
LC 743 strain isolated from raw milk		10^8	120.11±21.07	2163.59±35.48	ND
		10^9	1160.38±57.44	3310.29±33.21	54.35±3.43

*The control (medium) level of IL-1α, TNF α and NO: 20.53±4.94, 46.12±8.04 and ND.
[1)]ND: Not determined.

centration of IL-1α, TNF-α and NO production was increased from 1160.38 pg/mL, 3310.29 pg/mL and 54.35 μM to 1395.37 pg/mL, 5034.15 pg/mL and 68.22 μM, respectively, by the cultivation with 10^9 CFU/mL sample.

Identification and DNA sequencing of selected strain LC743

It was examined the physiclogical and biochemical test to determine genus and species of selected LC743 strain. Selected LC743 strain was non-spore, cocci type, homo fermentive, 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 *Streptococcus* (Table 3).

Identification using the 16S rDNA sequencing method by the PCR of universal primer was result in the *Streptococcus macedonicus* with the possibility of 99% (data not shown). Based upon the result of this study, it has named as a *Streptococcus macedonicus* LC743.

Growth of strain

The number of viable *S. macedonicus* LC743 was determined by serial 10-fold dilution in 0.1% peptone water. 50 μL (9.6×10^5/mL) of *S. macedonicus* LC743 was inoculated in 150 mL of 10% reconstituted skim milk. And then culture was incubated at 34°C, 37°C and 40°C for 24 h by checking 3 h term and highest growth rate was found at 40°C. The optimum growth temperature of *S. macedonicus* LC743 was 40°C and it has taken 18 h to reach the pH to 4.34 under this condition (Fig. 2).

Antibiotic tolerance

It is very important for the probiotic strain can survive in the antibiotic circumstance. Table 4 shows the tolerance of *S. macedonicus* LC743 strain on the 16 kinds of antibiotics. *S. macedonicus* LC743 showed more sensitive to novobiocin and oxacillin compared to 14 different antibiotics, and showed most resistance to gentamycin and lincomycin.

Lim *et al.* (2010) has reported that *L. paracasei* subsp. *paracasei* BFI46 was sensitive to penicillin-G and chlor-

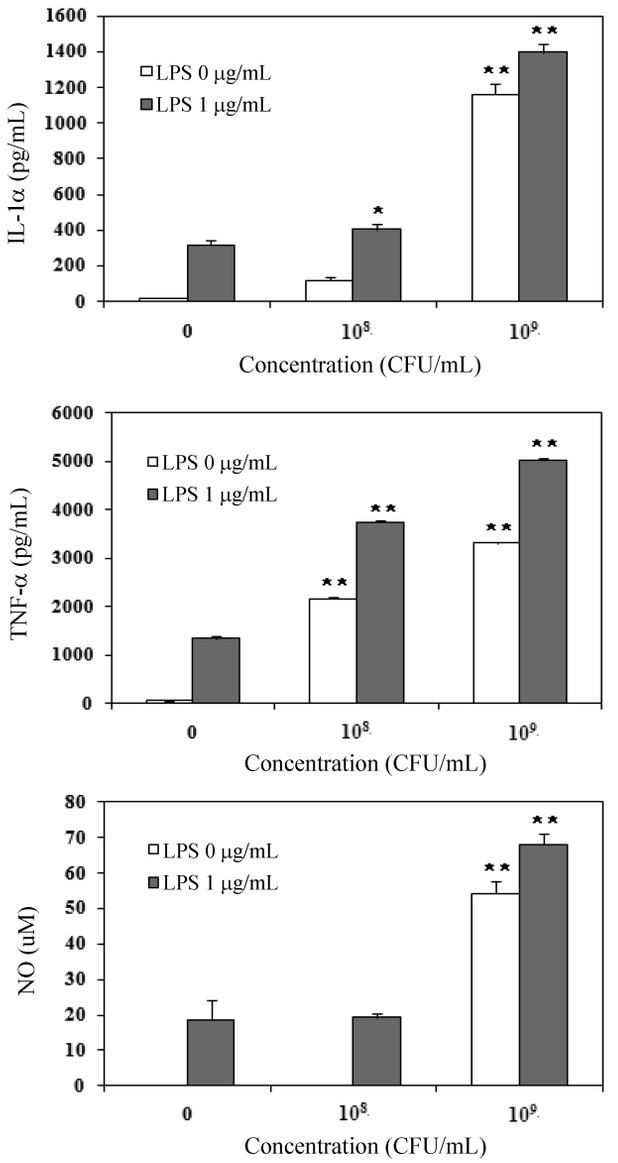

Fig. 1. Effect of *Streptococcus macedonicus* LC 743 on IL-1α, TNF-α and NO production by RAW 264.7 cells. *different significantly from control values (p<0.05). **different significantly from control values (p<0.01).

amphenicol but it shown high resistance to neomycin, kanamycin and polymycin.

Enzyme activity

Table 5 shows the enzyme activity of *S. macedonicus* LC743. The leucine arylamidase and acid phosphatase activity was 5 and it was relatively high on the other hand β-glucuronidase activity of carcinogen-producing enzymes in the gut was negative and there was no enzyme activity. This result is similar to the report of Lim *et al.* (2010) on the β-glucuronidase activity of *L. paracasei* subsp. *paracasei* BFI46.

Table 3. Physiological characteristics of *Streptococcus macedonicus* LC743

Gram reaction	+
Cell type	cocci
Spore forming	-
Motility	-
Aerobic growth	+
Anaerobic growth	+
Catalase reaction	-
Growth at 15°C	-
Growth at 45°C	+
Gas forming from glucose	-
Ammonia production from arginin	-
Acid production from	
Sodium pyruvate	+
Hippuric acid	-
Esculin ferric citrate	-
Payroglutamic acid-β-naphthylamide	-
6-Bromo-2-naphthyl-αD-galactopyranoside	+
Naphthol ASBI-glucuronic acid	-
2-Naphthyl-phosphate	-
L-Leucine-β-naphthylamide	+
L-Arginine	-
D-Ribose	-
L-Arabinose	-
D-Mannitol	-
D-Sorbitol	-
D-Lactose (bovine origin)	+
D-Trehalose	-
Inulin	-
D-Raffinose	-
Starch	-
Glycogen	-

Bile tolerance

Fig. 3 shows growth curves in MRS broth or MRS broth containing 0.3% bile. The log value of population after 7 h incubation without 0.3% oxgall was 9 but 8.4 with the addition of 0.3% bile so that it has shown good bile tolerance. This result is similar 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).

Acid tolerance

Acid tolerance is a property that the stain could survive in GI tract (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 small intestine through the

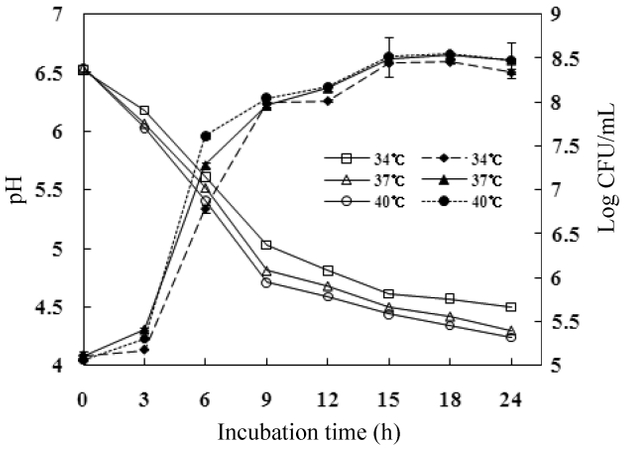

Fig. 2. Growth and pH changes of *Streptococcus macedonicus* LC743 in 10% reconstituted skim milk at various temperatures.

Table 4. Antibiotic susceptibility of *Streptococcus macedonicus* LC743

Antimicrobial agents	Minimal inhibitory concentrations (μg/mL)
Aminoglycosides	
Amikacin	640
Gentamycin	1280
Kanamycin	400
Neomycin	400
Streptomycin[1)]	400
β-lactams	
Penicillin-G[1)]	20
Methicillin	160
Oxacillin	4
Ampicillin	640
Gram-positive spectrum	
Bacitracin[1)]	10
Rifampicin	7.5
Novobiocin	2.5
Lincomycin	800
Gram-negative spectrum	
Polymyxin B[1)]	75
Broad spectrum	
Chloramphenicol	80
Vancomycin	100

[1)]Units/mL.

stomach (Booth, 1985; Mcdonald *et al.*, 1990). The acid tolerance of lactic acid bacteria has been linked to the 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 different H^+-ATPase activity in the probiotics.

Fig. 4 shows the pH tolerance of *S. macedonicus* LC743. At the pH 2, The log value of population had decreased from 7.14 to 5.33 after 3 h incubation. At the pH 3, The

Table 5. Enzyme patterns of *Streptococcus macedonicus* LC743[1)]

Enzyme	Streptococcus macedonicus LC743
Alkaline phosphatase	0
Esterase (C4)	0
Esterase lipase (C8)	0
Lipase (C14)	1
Leucine arylamidase	5
Valine arylamidase	2
Cystine arylamidase	2
Trypsin	0
Chymotrypsin	0
Acid phosphatase	5
Naphthol-AS-BI-phosphohydrolase	3
α-galactosidase	3
β-galactosidase	0
β-glucuronidase	0
α-glucosidase	1
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.

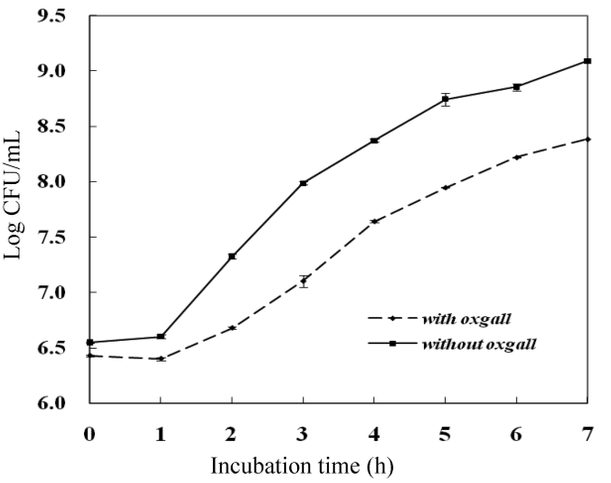

Fig. 3. Growth curve of *Streptococcus macedonicus* LC743 in MRS broth containing 0.05% L-cysteine with and without 0.3% oxgall.

log value of population had slightly decreased from 7.14 to 7.05 after 3 h incubation but it was higher than that at pH 2. As the test pH was increased to pH 4 and pH 6, it has shown no change. *S. macedonicus* LC743 is slightly influenced by low pH. But it has shown tolerance at high pH compared to the result of Jeon *et al.* (2007) that the

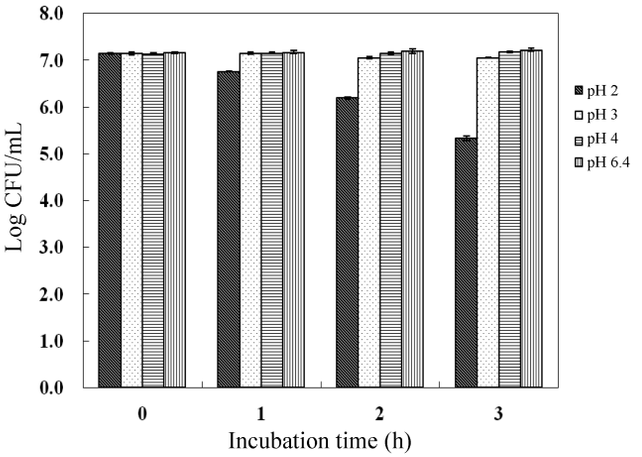

Fig. 4. Survival of *Streptococcus macedonicus* LC743 after 3 h in HCl solution (pH 2, 3, 4, 6.4).

cell density of 5.0×10^6 CFU/mL at pH 3 was decreased to 1.7×10^1 CFU/mL after 3 h incubation.

Antimicrobial activity

The antimicrobial ability is one of important property. The antimicrobial activity of lactic acid bacteria may be due to a number of factors. Among them, there could be factors including decreased pH levels, competition for the substances with a bacteridial or bacteriostic action, with bacteriocins (Parente and Ricciardi, 1999). Table 6 shows the antimicrobial activity of *S. macedonicus* LC743 against the pathogenic strains. *S. macedonicus* LC743 showed high resistence against *E. coli, S. Typhimurium* and *S. aureus* with the rate of 80.00%, 68.42%, and 81.54% respectively. The pH of media of pathogenic strain was 6.40-6.41 on the other hand, the pH of mixed strain media of pathogenic strain and *S. macedonicus* LC743 was 5.61-5.96 due to the acid production of *S. macedonicus* LC743. Lim *et al*. (2009) has reported that *L. acidophilus* RMK567 has antimicrobial activity on the *E. coli, S. Typhimurium* and *S. aureus* with the rate of 29.21%, 39.06% and 51.40% on the other hand, *S. macedonicus* LC743 shown high antimicrobial activity on *E. coli, S. Typhimurium* and *S. aureus.*

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Table 6. Inhibition of pathogens by *Streptococcus macedonicus* LC743 in the MRS broth

Pathogens[2)]	Growth[1)]				Inhibition (%)
	Pathogens[a]		*Streptococcus macedonicus* LC743[3)] + Pathogens		
	CFU/mL	pH	CFU/mL	pH	
Escherichia coli	7.0×10^6	6.41	1.4×10^6	5.66	80.00
Salmonella Typhimurium	9.5×10^6	6.40	3.0×10^6	5.96	68.42
Staphyloccous aureus	1.3×10^7	6.41	2.4×10^6	5.61	81.54

[1)]Initial count of *Streptococcus macedonicus* LC743: 5.0×10^6 CFU/mL.
[2)]Initial count of *E. coli, S. Typhimurium* and *S. aureus*: 9.8×10^5CFU/mL, 1.2×10^6 CFU/mL and 1.9×10^6 CFU/mL, respectively.
[3)]Determined after 6 h of incubation at 37°C.

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