Korean J. Food Sci. Ani. Resour.
Vol. 30, No. 2, pp. 223~231(2010)

ARTICLE

Physiological Characteristics and Immunomodulating Activity by *Lactobacillus paracasei* subsp. *paracasei* BFI46 Isolated from New-Born Infant Feces

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Abstract

In order to develop a new starter for fermented milk, *Lactobacillus paracasei* subsp. *paracasei* BFI46 (BFI46) obtained from new-born infant feces was investigated for physiological characteristics. Good immunomodulating activity was evident compared with commercial lactic acid bacteria starter cultures. The optimum growth temperature of BFI46 was 40°C with 12 h required to reach pH 4.3. Testing with 13 different antibiotics revealed greatest sensitivity of BFI46 to penicillin-G and chloramphenicol, and heightened resistance to neomycin, kanamycin and polymyxin. BFI46 displayed higher esterase activities compared to 18 other enzymes, was comparatively tolerant to bile juice and able to survive at pH 2 for 3 h, and displayed high resistance against *Escherichia coli* and *Salmonella Typhimurium* with a survival rate of 57.14% and 96.36%, respectively. The results indicate that BFI46 could be an excellent starter culture for fermented milk with high level of immunomodulating activity.

Key words: *Lactobacillus paracasei* subsp. *paracasei*, immunomodulating activity, physiological characteristics, fermented milk

Introduction

Numerous studies have investigated the therapeutic effects of fermented dairy products and lactic acid bacteria in cancer (Hirayama and Rafter, 2000; Liu *et al*., 2002, 2005; Yasutake *et al*., 2000), allergy (Liu *et al*., 2006; Matsuzaki *et al*., 1998; Shida *et al*., 2002), infection and gastrointestinal disorders (Adolfsson *et al*., 2004). Since the immune system is involved in most of these diseases, an immune-stimulatory effect of those products and lactic acid bacteria (LAB) was evaluated by *in vitro* or *in vivo* tests. Animal studies showed that LAB exerts antitumor effects. Some LAB, such as *Lactobacillus rhamnosus* GG (Goldin *et al*., 1996), *Lactobacillus acidophilus* (Goldin and Gorbach, 1977; Lidbeck *et al*., 1992), *Bifidobacterium longum* (Reddy and Rivenson, 1993), *Lactobacillus casei* strain Shirota (Yasutake *et al*., 2000) and components of LAB (Shiomi *et al*., 1982) were shown to exert tumor-suppressing effects. For reduction of allergy, Matsuzaki *et al*. (1998) reported that oral feeding of *Lactobacillus casei* strain Shirota effectively inhibited Immunoglobulin(Ig)E production both *in vivo* and *in vitro*. Besides stimulation of antibody production (Alvarez-Olmos and Oberhelman, 2001) and macrophage activity (Schiffrin *et al.*, 1995), functional effects like inhibition of inflammation (Hart *et al*., 2003; Chapat *et al*., 2004), and autoimmune disorders (Gill and Guarner, 2004) were observed.

The immune-stimulatory effect of fermented dairy products, especially yoghurt, is believed to be due to bacterial components (Meydani and Ha, 2000). The cell wall components (Maragkoudakis *et al*., 2006) and cytoplasms of some LAB strains (Meydani and Ha, 2000) have been shown to have immune-stimulatory properties. These bacterial components are recognized by corresponding toll-like receptors (TLR), followed by cytokine production (Takeda and Akira, 2005). TLR family members are transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions (Faure *et al*., 2000). The different TLRs recognize a broad spectrum of highly conserved microbial structures. It has been reported that the Gram-negative bacteria compound lipopolysaccharide (LPS), is recognized by TLR-4 (Karlsson *et al*., 2004). TLR-2 recognizes a variety of microbial components

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including peptidoglycans and lipoproteins from both Gram-positive and Gram-negative bacterial cell walls (Galdeano and Perdigon, 2006). In addition to LAB, fermented milk products contain other non-bacterial components produced during fermentation that also contribute to immunogenicity (de LeBlanc *et al*., 2006). Peptides (LeBlanc *et al*., 2002) and water soluble polysaccharides (Vinderola *et al*., 2006) have been shown to enhance the immune response.

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

Materials and Methods

Isolation of lactic acid bacteria

Fifity five new-born infant feces were collected under support of Soon Chun Hyang Hospital and Hangang Sungshim Hospital. Strain BFI46 was isolated from infant feces in Modified MRS medium (Table 1). The strain was incubated in lactobacilli MRS broth as the growth medium at 37°C for 24 h.

Identification of strain BFI46

The properties of the strain BFI46 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. The API 50 CHL-kit, an identification system for microorganisms, was used to investigate and identify the assimilation of the 49 carbohydrates. BFI46 strain, tentatively identified as *Lactobacillus paracasei* subsp. *paracasei* in the above tests, 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).

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

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).

Preparation of lactic acid bacteria

All organisms were inoculated in MRS broth, and then incubated at 37°C for 12 h (until entry about 10^9cells/mL). Cells were collected by centrifugation at 1,500 *g*, for 15 min, washed once with a physiological saline solution, and resuspended in 1.5 mL a Hanks' buffered salt solution. For addition to cell cultures, lactic acid bacteria were heat killed at 100°C for 50 min, as described by Marin *et al*. (1997).

Nitric oxide production

Sample heated as described above and RAW264.7 (monocyte; macrophage, mouse, 2×10^5cells/mL) obtained from Korean Cell Line Bank in 96-well plate were incubated at 37°C for 48 h in the CO_2 incubator, and nitric oxide 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 540nm 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 (Thermo Scientific; Rockford, IL, USA). IL-1α was determined by using the following procedure; 50 μL of Biotinylated antibody reagent was added to each well. Fifty μL of standards or samples was added to each well in duplicate. Plate was covered and incubated at 25°C for 2 h. Liquid was removed and plate was washed 3 times. A hundred μL of prepared Streptavidin-HRP Solution was added to each well. Plate was covered and incubated at room temperature for 30 min. Plate was washed 3 times. A hundred μL of 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution was added to each well. Plate was developed in the dark at 25°C for 30 min. Reaction was stopped by adding 100 μL of 1 N 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; 50 μL of standards or samples was added to each well in duplicate. 50 μL of Biotinylated antibody reagent was added to each well. Plate was covered and incubated at 25°C for 2 h. Liquid was removed and plate was washed 5 times. Streptavidin-HRP Concentrate was diluted. A hundred μL was added to each well. Plate was covered and incubated at 25°C for 30 min. Plate was washed 5 times. A hundred μL of 3,3',5,5'-tetramethylbenzidine (TMB) Substrate was added to each well. Plate was developed in the dark at 25°C for 30 min. Reaction was stopped by adding 100 μL of 1 N 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 *L. paracasei* subsp. *paracasei* BFI46 was determined by serial 10-fold dilution in 0.1% peptone water. *L. paracasei* subsp. *paracasei* BFI46 was inoculated 50 μL (9.6×10^5/mL) into 150 mL of 10% reconstituted skim milk. And then culture was incubated at 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. paracasei subsp. *paracasei* BFI46 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. paracasei* subsp. *paracasei* BFI46 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. paracasei* subsp. *paracasei* BFI46 was grown at 37°C for 18 h on MRS broth. Culture of *L. paracasei* subsp. *paracasei* BFI46 were compared for their ability to grow in the presence of bile by individual inoculation (1%) into sterile MRS broth containg 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. paracasei* subsp. *paracasei* BFI46 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 of 2.0, 3.0, and 4.0. Sterile double-distilled water (pH 6.4) served as the control. Ten mL of each pH solution were transferred into sterile test tubes.

One mL of stock culture containing approximately 10^9 CFU/mL of *L. paracasei* subsp. *paracasei* BFI46 using MRS agar containing 0.05% cysteine was then transferred into each of the four pH solutions. The pH solutions containing *L. paracasei* subsp. *paracasei* BFI46 were then incubated anaerobically at 37°C, followed by intermittent plating after 1, 2, and 3 h to stimulate survival of *L. paracasei* subsp. *paracasei* BFI46 under pH conditions common to the human stomach. *L. paracasei* subsp. *paracasei* BFI46 after each storage interval was

incubated anaerobically at 37°C for 48 h using MRS agar.

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 for 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 and selection of lactic acid bacteria

Fifty five new-born infant feces were collected under the support of Soon Chun Hyang Hospital and Hangang Sungshim Hospital. Two hundred one strains were isolated as a lactic acid bacteria from infant feces in modified MRS medium (Table 1). Among them 153 strains were selected by the incubation in the 10% reconstituted skim milk at 37°C for 24 h and reach the pH to 4.3 and coagulated.

Selection of strain of immunomodulating activity

The strain coagulated skim milk by the incubation at 37°C for 24 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. BFI46 strain were selected by the succination 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 continued by the IL-1α, TNF 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 activated macrophages and many other different types of cells including fibroblasts, T cells, B cells, astrocytes, and keratinocytes (Dinarello, 1991). TNF is also produced by activated macrophages, fibroblasts, and many different types of cells. The regulation of IL-1α and TNF 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 immunopotentiate the gut mucosal immune system via activation

Table 2. Immunomodulating activity produced after incubation at 37°C for 24 h in the 10% reconstituted skim milk added 1% lactic acid bacteria

Strains	Source	IL-1α (pg/mL)	TNF (pg/ mL)	NO (μM)
ABT-L*[1)]	Rhodia Inc.	491.15±31.86	>2450	18.35± 0.36
*Lactobacillus acidophilus**	Rhone-poulenc	24.26± 3.81	>2450	17.68± 0.73
ABT-D*[1)]	Rhone-poulenc	394.63± 5.37	>2450	18.53± 1.04
*Bifidobacterium longum**	Rhone-poulenc	558.25±99.22	>2450	20.08± 0.26
ST-1(*Streptococcus thermophillus*)*	Culture Sytems, Inc.	303.41±68.56	>2450	17.70± 0.66
ST-5(*Streptococcus thermophillus*)*	Culture Sytems, Inc.	164.65±10.74	>2450	16.70± 0.51
*Lactobacillus acidophilus**	Culture Sytems, Inc.	121.56± 9.42	>2450	14.50± 0.23
Lactobacillus acidophilus	ATCC 11506, IFO 3205	722.20±16.40	>2450	23.91± 2.53
Lactobacillus cremoris	KFRI 00349	736.23±10.02	>2450	18.40± 3.40
Lactobacillus delbrueckii subsp. *lactis*	ATCC 7830, FO 3376	815.39± 9.08	>2450	18.14± 3.05
Lactobacillus bulgaricus	ATCC 33409	740.53± 2.39	>2450	11.22± 1.13
Lactobacillus amylophilus	NRRL B-4437	835.25± 4.11	>2450	18.20± 3.77
Lactobacillus reuteri	KFRI 00661	736.23± 2.02	>2450	28.30±11.23
BFI46 strain isolated from new-born infant feces		>1000	>2450	19.58± 0.61

*Commercial lactic acid bacteria.

[1)]ABT-L, ABT-D : Mixed culture constituted of *Lactobacillus acidophilus, Bifidobacterium longum* and *Streptococcus thermophilus*.

Each data value indicates the mean±SD (n=3).

of macrophages and production of cytokines (Perdigon *et al*, 1992). Increased secretion of interleukin-1 and tumor necrosis factor (TNF)-α by peritoneal macrophages occurs after intraperitoneal exposure to *Bifidobacterium longum*, or *B. animalis* and *Lactobacillus bulgaricus*, or *Streptococcus thermophilus* (Solis Pereyra *et al.* 1991). In addition to cytokines, leukocytes can produce reactive oxygen intermediates involved in immune response to invading microorganism. One of the most important of the mediators is nitric oxide (Tejada-Simon *et al.*, 1999)

Table 2 shows the comparison of immunomodulating activity of selected and commercial strains and immunomodulating test sample was prepared by the autoclaving the cultivated lactic acid bacteria at 37°C for 24 h in the skim milk medium. The selected BFI46 strain was proven to be an excellent immunomodulating activity by the high interlukin -1α secretion, NO secretion, and TNF secretion activity.

Gill (1998) reported that IL-1α stimulate T- and B-cell proliferation. This finding indicated that the immunogenicity of lactic acid bacteria differs depending on the properties of a particular strain rather than on the common characteristics of the species. Cross (2002) also reported that the host response to stimuli by LAB was dependent on the strains of bacteria used. Different lactobacilli are able to differentially modulate the immune response (Cross *et al*., 2004; Tejada-Simon and Pestka, 1999).

Identification and DNA sequencing of selected strain BFI46

The physiological and biochemical test was proceed to determine genus and species of selected BFI46 strain. Selected BFI46 strain was non-spore, rod type, homo fermentive, gram positive bacteria and exhibited negative properties on catalase and motility. Also, it can grow at 15°C and 45°C. It do not produce gas and ammonia from glucose and arginine so that it was identified as a genus *Lactobacillus.*

The API 50 CHL-kit, an identification system for microorganisms, was used to investigate and identify the assimilation of the 49 carbohydrates. BFI46 strain, tentatively identified as *Lactobacillus paracasei* subsp. *paracasei* through the API 50 CHL-kit tests, with the possibility of 99.3% (Table 3). Identification using the 16S rDNA sequencing method by the PCR of universal primer was result in the *L. paracasei* subsp. *paracasei* with possibility of 99% (data not shown). Based upon the result of previous study, it was named as a *Lactobacillus paracasei* subsp. *paracasei* BFI46.

Table 3. Physiological characteristics of *L. paracasei* subsp. *paracasei* BFI46

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 arginin	-		
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 *L. paracasei* subsp. *paracasei* BFI46

The number of viable *L. paracasei* subsp. *paracasei* BFI46 was determined by serial 10-fold dilution in 0.1% peptone water. Fifty μL (9.6×10^5/mL) of *L. paracasei* subsp. *paracasei* BFI46 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 the highest growth rate was found at 40°C. The optimum growth temperature of *L. paracasei* subsp. *paracasei* BFI46 was 40°C and it has taken 12 h to reach the pH to 4.3 under this condition (Fig. 1).

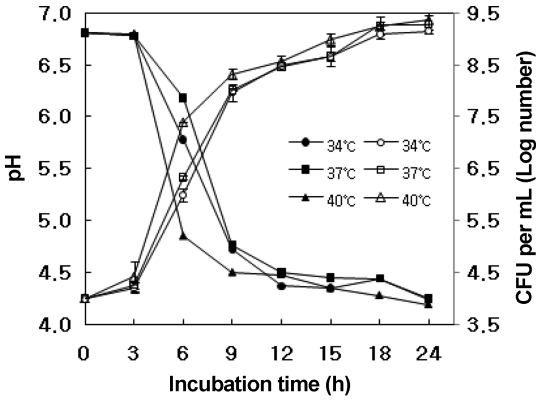

Fig. 1. Growth and pH change of *L. paracasei* subsp. *paracasei* BFI46 in 10% reconstituted skim milk at various temperatures.

Antibiotic tolerance of *L. paracasei* subsp. *paracasei* BFI46

It is very important for the probiotic strain can survive in the antibiotic circumstance. Table 4 shows the tolerance of *L. paracasei* subsp. *paracasei* BFI46 strain on the 15 kinds of antibiotics. *L. paracasei* subsp. *paracasei* BFI46 showed more sensitive to penicillin-G and chloramphenicol in a comparison of 13 different antibiotics, and showed most resistance to neomycin, kanamycin, and polymyxin.

Table 4. Antibiotics susceptibility of *L. paracasei* subsp. *paracasei* BFI46

Antimicrobial agents	Minimal inhibitory concentrations (mg/mL)
Aminoglycosides	
Amikacin	640
Gentamycin	5120
Kanamycin	9600
Neomycin	9600
Streptomycin*	4000
β-lactams	
Penicillin-G*	20
Methicillin	320
Oxacillin	240
Ampicillin	1280
Gram-positive spectrum	
Bacitracin*	120
Rifampicin	240
Novobiocin	90
Lincomycin	800
Gram-negative spectrum	
Polymyxin B*	16000
Broad spectrum	
Chloramphenicol	40

* : units/mL

Lim *et al.* (2009) has reported that *Lactobacillus acidophilus* RMK567 was sensitive to penicillin-G and novobiocin with the level of 20 μg/mL and 30 ug/mL but it has shown high resistance to kanamycin, neomycin, and streptomycin with the level of 1600 μg/mL.

Enzyme activity of *L. paracasei* subsp. *paracasei* BFI46

Table 5 shows the enzyme activity of *L. paracasei* subsp. *paracasei* BFI46. The esterase activity was 4 and it was relatively higher than other enzymes on the other hand β-galactosidase activity was 2 and it was relatively lower than other enzymes. This result is relatively low level of β-galactosidase activity compared to the report of Lim *et al.* (2009) on the β-galactosidase activity of *Lactobacillus acidophilus* RMK567.

Bile tolerance of *L. paracasei* subsp. *paracasei* BFI46

Fig. 2 shows the bile tolerance of *L. paracasei* subsp. *paracasei* BFI46 strain. The log value of population after 7 h incubation without 0.3% oxgall was 9 but 8.5 with the

Table 5. Enzyme patterns of *L. paracasei* subsp. *paracasei* BFI46 (*)

Enzyme	*L. paracasei* subsp. *paracasei* BFI46
Alkaline phosphatase	3
Esterase(C4)	4
Esterase lipase(C8)	3
Lipase(C14)	1
Leucine arylamidase	1
Valine arylamidase	1
Cystine arylamidase	1
Trypsin	2
Chymotrypsin	1
Acid phosphatase	3
Naphthol-AS-BI-phosphohydrolase	3
α-Galactosidase	0
β-Galactosidase	2
β-Glucuronidase	2
α-Glucosidase	1
β-Glucosidase	1
N-Acetyl-β-glucosaminidase	2
α-Mannosidase	2
α-Fucosidase	2

*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.

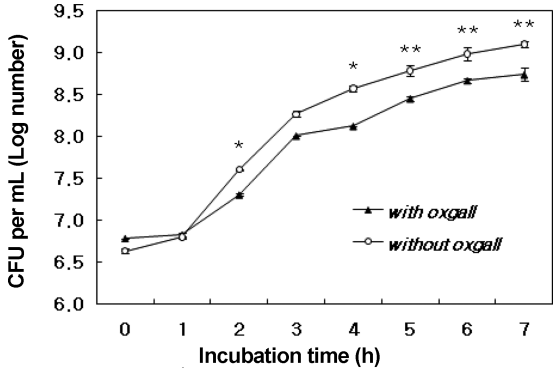

Fig. 2. Growth of *L. paracasei* subsp. *paracasei* BFI46 in MRS broth containing 0.05% L-cysteine with or without 0.3% oxgall (*p<0.05, **p<0.01).

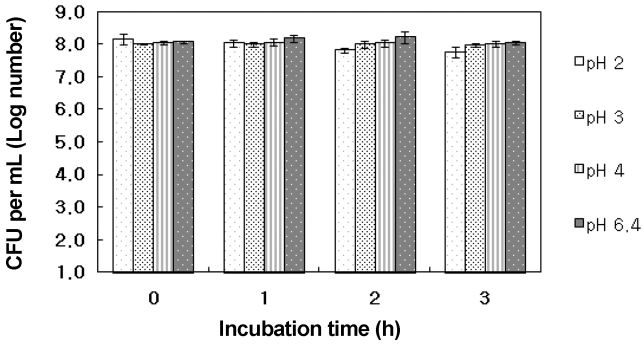

Fig. 3. Survival of *L. paracasei* subsp. *paracasei* BFI46 after 3 h in HCl solution (pH 2, 3, 4, 6.4).

addition of 0.3% oxgall so that it has shown good bile tolerance in spite of the significant difference. This result is similar trends to the report of Lim *et al*. (2009) on the bile tolerance of *Lactobacillus acidophilus* RMK567 that it has shown slight decrease in the value of OD_{610} from 1.511 to 1.127.

pH tolerance of *L. paracasei* subsp. *paracasei* BFI46

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 stomach (Booth, 1985; Mcdonald *et al.*, 1990). Fig. 3 shows the pH tolerance of *L. paracasei* subsp. *paracasei* BFI46. It could be recognized that the *L. paracasei* subsp. *paracasei* BFI46 has pH tolerance by the cell density of pH 2 and 3 was similar to that of pH 6.4. And this result was similar to the report of Lim *et al.* (2009) on the *L. acidophilus* RMK567. But it has shown high pH tolerance compared to the result of Jeon *et al.* (2007) that the cell density at pH 3 of 5.0×10^6 cfu/mL was decreased to 1.7×10^1 CFU/mL after 3 h.

Antimicrobial activity of *L. paracasei* subsp. *paracasei* BFI46

Table 6 shows the antimicrobial activity of *L. paracasei* subsp. *paracasei* BFI46 against the pathogenic strains. *L. paracasei* subsp. *paracasei* BFI46 showed high resistence against *Escherichia coli and Salmonella Typhimurium* with the rate of 57.14% and 96.36% respectively. Whereas it didn't show antimicrobial activity against *Staphylococcus aureus.*

The pH of media of pathogenic strain was 6.41-6.44 on the other hand, the pH of mixed strain media of pathogenic strain and of *L. paracasei* subsp. *paracasei* BFI46 was 4.74-5.09 due to the acid production of of *L. paracasei* subsp. *paracasei* BFI46. Lim *et al*. (2009) has reported that *L. acidophilus* RMK567 has antimicrobial activity on the *Escherichia coli, Salmonella Typhimurium* and *Staphylococcus aureus* with the rate of 29.21%, 39.06% and 51.40% on the other hand, *L. paracasei* subsp. *paracasei* BFI46 shown high antimicrobial activity on *Escherichia coli* and *Salmonella Typhimurium* but low antimicrobial activity on the *Staphylococcus aureus.* And it is superior to the report of Lim *et al.* (2008) that *L. zeae* RMK354 has antimicrobial activity on the *Salmonella Typhimurium* with the rate of 60.0% but no antimicrobial activity to 2 other pathogenic bacteria.

Table 6. Inhibition of pathogens by *L. paracasei* subsp. *paracasei* BFI46 in MRS broth

Pathogens	Growth				Inhibition (%)
	Pathogens[a]		*L. paracasei* subsp. *paracasei* BFI46[a] + Pathogens		
	CFU/mL	pH	CFU/mL	pH	
Escherichia coli	2.8×10^7	6.41	1.2×10^7	4.75	57.14
Salmonella Typhimurium	1.1×10^7	6.44	4.0×10^5	5.09	96.36
Staphyloccous aureus	2.8×10^6	6.42	5.3×10^7	4.74	0

* Initial count of *L. paracasei* subsp. *paracasei* BFI46 : 1.1×10^6CFU/mL.
[a] Determined after 6 h of incubation at 37°C.

Acknowledgments

The study was supported by Ministry for Food, Agriculture, Forestry and Fisheries, 2006. The authors were also partially supported by Technology development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, 2007.

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(Received 2009.11.3/Revised 2010.4.6/Accepted 2010.4.6)