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Characterization of *Lactobacillus cellobiosus* D37 Isolated from Soybean Paste as a Probiotic with Anti-Cancer and Antimicrobial Properties

Sung-Mee Lim*, Goon-Ja Lee, Sun-Mee Park[1], Dong-Hyun Ahn[2], and Dong-Soon Im[3]

Department of Food Science and Technology, Tongmyong University, Busan 608-735, Korea
[1]Epidemiology Division, Busan Metropolitan City Institute of Health and Environment, Busan 613-806, Korea
[2]Department of Food Science and Technology, Pukyong National University, Busan 608-737, Korea
[3]College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Korea

Abstract The probiotic characteristics of a total of 137 lactic acid bacterial strains isolated from soybean paste were investigated. Among those tested, the D37 strain was selected as a probiotic bacteria due to its acid and bile tolerance, and its strong anti-cancer and antibacterial activities. The D37 strain showed highly stable viability at acidic pH for 2 hr, and was very stable in 10% bovine bile. The viability of human colon cancer HT-29 cells was inhibited more than 60% at a 200 μL/mL concentration of D37 cell-free culture supernatant, and the degree of inhibition was concentration-dependent. The D37 strain showed a wide range of antibacterial activities against food-borne pathogenic bacteria such as *Escherichia coli* O157, *Listeria* spp., *Vibrio* spp., *Salmonella* spp., and *Staphylococcus aureus*. According to phenotypic characteristics and the utilization of various sugars, the D37 strain was identified as *Lactobacillus cellobiosus*.

Keywords: soybean paste, acid and bile tolerance, anti-cancer activity, antimicrobial activity, *Lactobacillus cellobiosus*

Introduction

Colon cancer is one of the leading causes of cancer morbidity and mortality in Western countries, including the United States (1). As the Korean lifestyle has quickly become more westernized, the number of patients with colorectal cancer and the associated mortality rate have been increasing. Recently, much attention has been focused on decreasing cancer risk through dietary alterations, particularly by increasing the intake of dietary fiber. There is also a potential role for foods that contain probiotics to change the colonic microflora in a way that might prevent diseases such as colorectal cancer (2, 3).

The term 'probiotics' refers to live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance (4). Probiotics are health enhancing functional food ingredients used therapeutically to prevent or shorten the duration of antibiotic- and rotavirus-associated diarrhea, alleviate lactose intolerance, and modulate immune function (5, 6). Some evidence suggests that probiotic bacteria such as *Lactobacillus, Streptococcus, Leuconostoc,* and *Bifidobacterium* spp. may also have the potential to lower serum cholesterol levels and prevent colon cancer (7). The precise mechanisms by which lactic acid bacteria (LAB) may inhibit cancer are currently unknown. Such mechanisms might include the modification of physicochemical conditions in the gut, increasing the net production rate of short chain fatty acids (SCFA), binding and degrading potential carcinogens, producing antitumorigenic or antimutagenic compounds, and enhancing the host's immune response. Furthermore, probiotics reduce the level of procarcinogenic enzymes such as β-glucuronidase, nitroreductase, and azoreductase (8).

Many LAB are active both *in vitro* and *in vivo* against a variety of Gram-positive pathogenic bacteria such as *Listeria monocytogenes, Clostridium botulinum, Vibrio cholerae,* and *Staphylococcus aureus* (9-12). Furthermore, *L. casei* Strain Shirota was effective in significantly reducing the levels of *Helicobacter pylori* colonization, and had a long-term suppressive effect on *H. pylori* in humans, which could help prevent gastric cancer (13). Probiotic bacteria can prevent the colonization of the gut by pathogenic microorganisms stimulate generation of the mucus barrier through the production of antimicrobial and antibacterial compounds (bacteriocin, hydrogen peroxide, lactic acid, and diacetyl), reduce the luminal pH through SCFA production, and compete for limited nutrients (14).

Probiotic bacteria are found mainly in fermented foods, and dairy products also play an important role as carriers of probiotics (15). They have considerable potential for inclusion in functional foods and health-related products. Also, Chang *et al.* (16) showed that the body weights of piglets fed diets containing probiotics were fatter than control piglets, which suggests that probiotic supplementation in piglets would be economical in pig farming. Organisms used as probiotics must have properties of acid and bile tolerance, the production of antimicrobial substances that inhibit pathogen growth, and antagonism toward carcinogenic microorganisms.

Yoo *et al.* (17) reported that *Bacillus* spp., mold, yeast, and LAB were dominant in soybean sauce and play an important role in soybean paste fermentation. Bacterial strains isolated from the traditional Korean soy paste, *doenjang*, showed inhibitory effects on angiotensin converting enzyme (ACE) and fibrinolytic activity (18). Moreover, methanol extracts and fractions from *doenjang* showed strong antioxidant and anticarcinogenic effects

*Corresponding author: Tel: 82-51-620-3428; Fax: 82-51-620-3649
E-mail: limsm020@tu.ac.kr
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toward A549 cells (human lung carcinoma), MCF-7 cells (human breast adenocarcinoma), and inhibited chemical mutagenesis (19, 20). However, the potential probiotic properties of LAB isolated from traditional Korean soybean pastes have rarely been reported. Therefore, to determine the potential uses of LAB as probiotic bacteria, we assessed the acid and bile tolerance, and the anti-cancer and antimicrobial activities of LAB isolated from the traditional Korean soybean paste, *doenjang*.

Materials and Methods

Media and reagents Lactobacillus MRS broth and Brain Heart Infusion (BHI) broth were purchased from the Difco Co. (Sparks, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Ltd., Paisley, UK). The API CHL 50 kit was obtained from the BioMërieux Co. (Marcy l'Etoile, France). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of LAB and preparation of cell-free culture supernatant (CFCS) Traditional Korean soybean paste (*doenjang*) was purchased from the local oriental grocery store in Busan. All samples were homogenized in sterile phosphate buffer solution (PBS, pH 7.2) for 5 min with a blender. Appropriate dilutions were prepared with PBS and 1 mL of serial 10-fold dilutions was spread onto Lactobacillus MRS agar plates containing 1% $CaCO_3$. Colonies forming clear zones on the MRS agar plate were selected. Cells were grown in MRS broth and harvested by centrifugation (7,000×g, for 10 min). The CFCS was then filtered through a 0.22 μm membrane filter (Millipore Corp., Billerica, MA, USA).

Cancer cell cultures HT-29 human colon adenocarcinoma epithelial cells were obtained from the Korean Cell Line Bank (KCLB). Cells were grown in 75 cm^2 tissue culture flasks in DMEM supplemented with 10%(v/v) FBS, 2 mM of glutamine, 1 mM of sodium pyruvate, 100 units/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified 5% CO_2 incubator until approximately 90% confluent.

Microorganism cultures *Bacillus* spp., *Lactobacillus* spp., *Enterococcus* spp., *Listeria* spp., *Streptococcus* spp., *Enterobacter aerogenes, Escherichia coli, Salmonella* spp., *Vibrio* spp., *Aspergillus oryzae, Penicillium roqueforti, Candida albicans,* and *Saccharomyces cerevisiae* were used as indicator microorganisms for the evaluation of antimicrobial activity. All strains were obtained from the ATCC (American Type Culture Collection), KCTC (Korean Collection for Type Cultures), or KCCM (Korean Culture Center of Microorganisms) and propagated in BHI broth at 37°C under aerobic conditions in order to reach the late logarithmic growth phase. Mold and yeast were cultured in potato dextrose agar (PDA) and YM agar at 25 °C, respectively.

Acid and bile tolerance of LAB To investigate LAB survival under acidic conditions, each strain of LAB was harvested by centrifugation (7,000×g, for 10 min), washed twice in PBS, inoculated (1%) into MRS broth acidified to pH 2.5 (using HCl) containing 1,000 units of pepsin (Sigma, St. Louis, MO, USA), or non-acidified MRS broth, and incubated at 37°C for 2 hr. The number of viable LAB cells was then determined by serial 10-fold dilution in PBS, and 0.1 mL aliquots were spread evenly on MRS agar. Plates were incubated aerobically at 37°C for 48 hr and the number of colony forming units (CFU) was estimated.

To estimate the bile tolerance of LAB, each strain was harvested by centrifugation (7,000×g, for 10 min), washed twice in PBS, and inoculated (1%) into MRS broth containing 1, 3, 5, and 10% bovine bile in 48 well plates. Growth of LAB in MRS broth without bovine bile served as a control. After incubation at 37°C for 24 hr, the absorbance was measured at 660 nm using an enzyme linked immunosorbent assay (ELISA) reader (Spectrocount, Packard Instruments, Meriden, CT, USA).

Inhibition of cancer cell viability HT-29 cells were resuspended in DMEM media with 0.5% FBS and incubated overnight with LAB bacteria or CFCS. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was then added followed by incubation for an additional 4 hr. The medium was removed and formazon crystals were dissolved in a DMSO/Ethanol 1:1 solution. Absorbance was measured at 570 nm using an ELISA reader. Individual histograms were made based on the percentage of the control level. MTT assays were conducted in triplicate.

Antimicrobial activity assay Antibacterial activity was tested against a wide spectrum of LAB as well as food-borne pathogens, yeast, and mold by the agar well diffusion assay (21). Appropriate agar (1.5%, w/v) media were inoculated (1%, v/v) with indicator microorganism suspension. After solidification, a sterilized pipette tip was used to punch an 8 mm well in each plate and 50 μL of CFCS was added to each well. The plates were incubated at optimal conditions and the diameter of the inhibition zones was measured.

Identification of LAB Selected LAB strains were characterized by physiological and biochemical tests according to the criteria of Bergey's Manual of Systematic Bacteriology (22). Cell morphology was determined by optical microscopy (DW-THN, Dongwon, Korea). Carbohydrate fermentation patterns were determined using the API 50 CH system (BioMerieux, France) according to the manufacturer's instructions.

Results and Discussion

Selection of acid and bile tolerant LAB A total of 137 LAB strains were isolated from soybean paste and screened for acid and bile tolerance. Among the isolated LAB, 19 strains were tolerant at acidic conditions (pH 2.5) and in 10% bovine bile (Table 1), whereas 118 strains were sensitive to the same conditions. The D11, D37, and D59 strains showed highly-stable viability in acidic pH for 2 hr, and the other strains exhibited relatively high survival

Table 1. Survival of LAB isolated from soybean paste in acidic pH and bovine bile

LAB	Condition[1]		Concentration of bovine bile[2] (%)				
	Nonacidified	Acidic pH	0	1	3	5	10
D08	8.62	7.58	+++	+++	+++	++	+
D11	8.74	8.04	+++	+++	+++	+++	++
D12	8.46	7.30	+++	+++	+++	+++	+++
D19	8.41	6.40	+++	+++	+++	+++	++
D21	8.49	7.08	+++	+++	+++	+++	++
D23	8.27	6.43	+++	+++	+++	+++	++
D37	8.40	8.19	+++	+++	+++	+++	+++
D38	8.23	7.64	+++	+++	+++	+++	++
D44	8.76	6.90	+++	+++	+++	+++	+
D49	8.73	6.76	+++	+++	+++	+++	++
D51	8.66	7.52	+++	+++	+++	+++	++
D59	8.54	8.45	+++	+++	+++	+++	+
D60	8.67	6.70	+++	+++	+++	+++	++
D66	8.58	6.79	+++	+++	+++	+++	+
D70	8.83	6.78	+++	+++	+++	+++	++
D74	8.64	7.75	+++	+++	+++	+++	+++
D78	8.72	6.23	+++	+++	+++	+++	++
D82	8.56	7.28	+++	+++	+++	+++	+++
D86	8.78	6.95	+++	+++	+++	+++	+++

[1]Viable cell counts (Log CFU/mL) of LAB after incubation at 37°C for 2 hr in nonacidified and acidic MRS broth.
[2]+++, O.D. value at 660 nm is more than 1.00; ++, 0.81 to 1.00; +, 0.51 to 0.80.

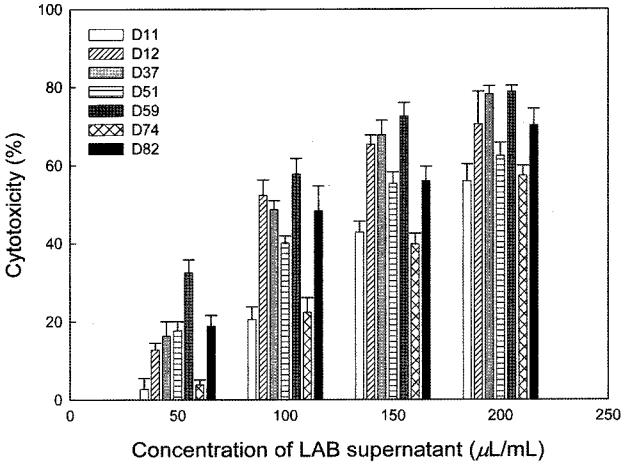

Fig. 1. Concentration-dependent inhibition of HT-29 cell viability by CFCS.

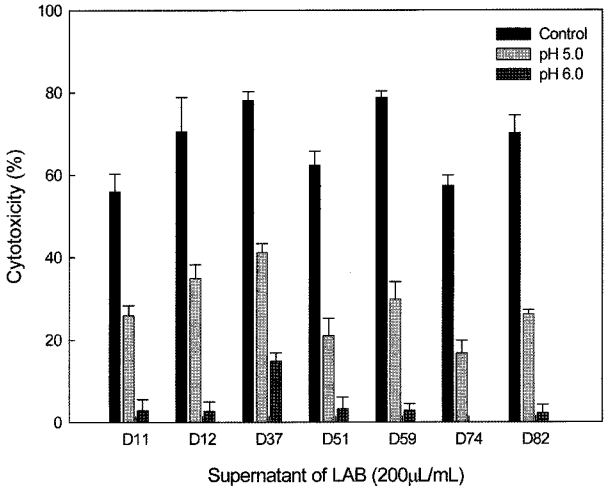

Fig. 2. pH dependence of CFCS-induced cytotoxicity toward HT-29 cells.

levels, although viable cell counts of the strains decreased by 2 logs. Meanwhile, most strains were stable in 5% bovine bile, and strains D12, D37, D74, D82, and D86 sustained high viability in 10% bile solution. Therefore, after passage through the stomach and the small intestine, these LAB strains may survive and become established transiently in the large bowel.

Probiotic bacteria should be resistant to the low pH of the stomach and the concentrations of bile, pancreatic juice and mucus found in the small intestine (23). *Lactobacillus* KM showed strong resistance to artificial gastric juice and artificial bile juice (24). Vinderola and Reinheimer (25) reported that *L. acidophilus* was the most interesting species since it showed high values of resistance to gastric juice and bile, and *B. bifidum* strains showed similar behavior, although the values of the parameters investigated were slightly lower than those obtained for *L. acidophilus*. In addition, *L. acidophilus* was the most acid-tolerant strain, while *L. casei* was the most acid-sensitive strain. Also, strains of *L. panis* and *L. pontis* showed prolonged survival at pH 2.5 in synthetic stomach juice and good growth in the presence of porcine bile salt (26).

Anti-cancer activity of LAB Focusing on LAB strains with relatively stable viability at pH 2.5 and in 10% bovine bile, we investigated the effect of LAB cells and CFCS on HT-29 cell viability. As shown in Fig. 1, CFCS of D11, D12, D37, D51, D59, D74, and D82 strains strongly inhibited the viability of HT-29 cells. The cytotoxicity was concentration-dependent, and the viability of HT-29 was inhibited more than 60% at a concentration of 200 μL/mL CFCS. At a CFCS concentration of 50 μL/mL, the inhibition rate of D59 was highest among the tested LAB strains. We considered the possibility that the anti-cancer activity of LAB is due to the low CFCS pH value, because the activity was not diminished by heating at 121°C for 15 min (results not shown). The CFCS pH values of strains D11, D12, D37, D51, D59, D74, and D82 incubated at 37°C for 18 hr were 4.57, 4.21, 3.98, 4.34, 3.83, 4.58, and 4.11, respectively. We found that CFCS with lower pH values showed higher anti-cancer activity. Meanwhile, the anti-cancer activity of CFCS from LAB was absent at neutral pH (Fig. 2). In general, anti-cancer activity was observed under pH 5.0, but the activity rapidly declined at pH 6.0 and disappeared at pH 7.0.

Viable D11, D37, and D51 cells also showed anti-cancer activity in a viable cell count-dependent manner, however, D12, D59, D74, and D82 cells did not affect the viability of HT-29 cells (Fig. 3). The anti-cancer activity of viable D37 cells was higher than that of D11 and D51. D37 showed cytotoxicity against HT-29 cells when the viable cells counts were as low as 10^5 CFU/mL, however D51 did not show cytotoxicity below 10^7 CFU/mL.

Lee *et al.* (27) reported that the cytoplasmic fractions of *L. casei* and *B. longum* were more effective at inhibiting cancer cell viability as shown by inhibition rates of approximately 50% at 50 μg/mL. Pool-Zobel *et al.* (28) suggested that *L. acidophilus, L. gasseri, L. confuses, S. thermophilus, B. breve,* and *B. longum* were also protective against 1,2-dimehtylhydrazine (DMH)-induced genotoxicity, and the peptidoglycan fraction and whole freeze-dried cells from *L. acidophilus* were antigenotoxic. Goldin *et al.* (29) investigated the hypothesis that diets enriched with *L.* GG can reduce colonic tumors in rats and concluded that the probiotics inhibited the initiation or early promotion of carcinogenesis. *L. casei* Shirota delayed

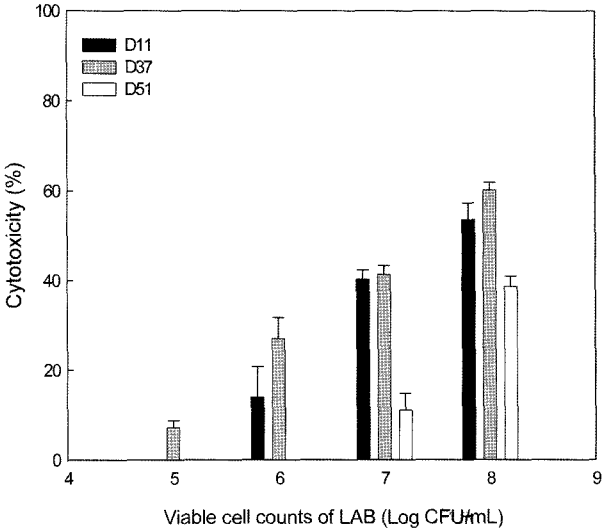

Fig. 3. Effects of viable LAB cell counts on the inhibition of HT-29 cell viability.

Table 2. Antimicrobial activity of unadjusted and neutralized CFCS of the D37 strain by the critical dilution method against various indicator microorganisms

Microorganisms	Indicator species	Inhibition zone[1] (mm)	
		Unadjusted	Neutralized
Gram-positive bacteria	*B. cereus* ATCC 11778	++	-
	B. subtilis ATCC 35421	++	-
	L. acidophilus KCTC 3168	-	-
	L. casei ATCC 25302	-	-
	L. paracaei ATCC 25302	-	-
	L. plantarum KCTC 1048	-	-
	E. faecalis KCTC 3206	++	-
	E. faecium KCCM 11028	+	-
	L. monocytogenes KCTC 3569	++	-
	L. innocua ATCC 33090	++	-
	L. ivanovii ATCC 19119	+++	-
	St. lactis ATCC 1913	-	-
	S. aureus ATCC 6538	++	-
Gram-negative bacteria	*Ent. aerogenes* ATCC 13480	++	
	E. coli ATCC 11229	++	-
	E. coli O157 ATCC 43889	+	-
	Sal. enteritidis ATCC 13076	++	-
	Sal. typhimurium KCTC 2514	++	-
	V. parahaemolyticus KCTC 2471	+++	-
	V. vulnificus KCTC 2982	++	-
Molds	*Asp. oryzae* KCTC 6983	-	-
	Pen. roqueforti KCCM 11269	-	-
Yeasts	*Ca. albicans* KCTC 7965	-	-
	Sa. cerevisiae KCTC 7246	-	-

[1]-, Inhibition zone not shown; +, 8.5 to 10.0 mm; ++, 10.1 to 12.0 mm; +++, more than 12.1 mm.

tumor onset and reduced tumor incidence in C3H/HeN mice, and the number of natural killer cells also increased in the *L. casei* Shirota treated group (30). *L. acidophilus* also inhibits aberrant crypt formation in mutagenized rats, indicative of activity that could decrease the risk of colon cancer (31). *In vitro* studies with *L. rhamnosus* GG and an *in vivo* study with *L. rhamnosus* GG and a *Propionibacterium* spp. showed a decrease in availability of carcinogenic aflatoxin in the lumen (32).

Antimicrobial activity of LAB For further examination, we selected the D37 strain because it showed the greatest acid and bile tolerance and anti-cancer activity. We next examined its antimicrobial activity against diverse indicator microorganisms. This strain was effective at inhibiting the growth of a wide range of diverse microorganisms including *Bacillus* spp. and food-borne pathogenic bacteria such as *Listeria* spp., *Salmonella* spp., *Vibrio* spp., *E. coli* O157, and *S. aureus*, but was not as effective with several LAB, yeasts, and molds, using unadjusted CFCS. The antimicrobial activity of D37 CFCS was the highest among the acid and bile tolerant LAB, although its pH value was higher than that of D59. MRS broth adjusted to the pH value of D37 CFCS using 1 N HCl (pH 3.98) showed a smaller inhibition zone on the agar plate against most indicator microorganisms than the D37 CFCS. In particular, no inhibition zone was seen against *E. faecium, E. faecalis,* and *E. coli* O157 (results not shown). Significantly, the D37 strain did not show inhibitory activity against these microorganisms with neutralized CFCS. We assumed that the D37 strain did not produce bacteriocin, bacterial proteins, or peptides that have bactericidal action against closely related species (33), however, the antibacterial activity of the strain may be due to the production of hydrogen peroxide, lactic acid, and diacetyl (2,3-butanedione).

L. casei strain Shirota or *L. acidophilus* exert growth inhibitory and bactericidal activities against shiga toxin-producing *E. coli* O157:H7 (34). Zhao *et al.* (35) demonstrated that selected probiotic bacteria administered to cattle prior to exposure to *E. coli* O157:H7 can reduce the level of *E. coli* O157:H7 carriage in most animals.

Table 3. Phenotypic characteristics and the utilization of various sugars by *L. cellobiosus* D37[1)]

Contents	Results	Sugar	Results	Sugar	Results
Cell shape	Rod	Glycerol	-	Salicine	-
Gram staining	+	Erythritol	-	Cellobiose	+
Spores staining	-	D-Arabinose	-	Maltose	+
Acid-fast staining	-	L-Arabinose	+	Lactose	-
Motility	-	Ribose	+	Melibiose	-
Gas from glucose	-	D-Xylose	-	Saccharose	+
H_2S production	-	L-Xylose	-	Trehalose	-
Lactic acid	L	Adonitol	-	Inuline	-
Nitrate reduction	-	β-Methyl-xyloside	-	Melezitose	-
Methyl red	+	Galactose	+	D-Raffinose	-
Voges-Proskauer	-	D-Glucose	+	Amidon	-
Horse blood hemolysis	-	D-Fructose	+	Glycogene	-
Sheep blood hemolysis	-	D-Mannose	+	Xylitol	-
Catalase	-	L-Sorbose	-	β-Gentiobiose	-
Oxidase	-	Rhamnose	-	D-Turanose	-
Urease	-	Dulcitol	-	D-Lyxose	-
Arginine hydrolysis	+	Inositol	-	D-Tagatose	-
Growth in aerobic condition	+	Mannitol	-	D-Fucose	-
anaerobic	+	Sorbitol	-	L-Fucose	-
Growth at 10-45°C	+	α-Methyl-D-mannoside	+	D-Arabitol	-
Growth at pH 5.0-9.0	+	α-Methyl-D-glucoside	-	L-Arabitol	-
10.0	-	N-Acetyl glucosamine	-	Gluconate	-
Growth in 1-5% NaCl	+	Amygdaline	-	2-ceto-gluconate	-
10% NaCl	-	Arbutine	-	5-ceto-gluconate	-
		Esculine	+		-

[1)]+, positive reaction; -, negative reaction; L, configuration of lactic acid produced from glucose.

Waard *et al.* (36) concluded that supplementation of *L. casei* significantly reduced the number of *L. monocytogenes* in the stomach, caecum, faeces, spleen, and liver 2 days after *L. monocytogenes* infection. Also, *L. rhamnosus* and *L. paracasei* subsp. *paracasei* inhibited the growth and enterotoxin production of *S. aureus* in sausages during fermentation (37). Lash *et al.* (38) reported that the CFCS of *L. plantarum* is effective at inhibiting the growth of a wide range of diverse microorganisms including *S. aureus*, *E. coli*, *L. innocua*, and *P. aeruginosa*. Two *Lactobacillus* strains isolated from swine and poultry showed similar results to the present study and were shown to inhibit the growth of *E. coli*, *S. typhimurium*, *S. aureus*, and *B. cereus* (39).

Identification of the D37 strain The D37 strain was catalase-negative, Gram-positive, facultatively anaerobic, rod-shaped with the ability to grow 10-45°C at pH 5.0-9.0, and tolerant to 5% salt. It could ferment glucose to L-lactic acid but did not produce gas, and produced ammonia from the hydrolysis of arginine. The D37 strain did not show urease or hemolysin activity toward horse and sheep blood. Meanwhile, the D37 strain could ferment galactose, fructose, mannose, and cellobiose, but not xylose, rhamnose, sorbitol, inuline, and raffinose (Table 3). The D37 strain was identified as *Lactobacillus cellobiosus* D37 based on these characteristics and the T=0.56 value obtained with the API 50 CH kit.

Some of the microorganisms commonly used to reinforce animal health and nutrition involve bacteria of the *Lactobacillus, Bifidobacterium, Streptococcus, Pediococcus, Enterococcus, Bacillus, Clostridium* genera, and yeasts and mold of the *Saccharomyces, Candida*, and *Aspergillus* genera. The functional properties of particular strains of lactic acid bacteria, such as *Lactobacillus* (*L. acidophilus, L. reuteri, L. brevis*), *Bifidobacterium* (*B. bifidum, B. thermophilus*), *Streptococcus* (*S. lactis*) have been extensively studied and well documented. In addition, these bacteria have traditionally been used in the production of fermented dairy products and are generally given GRAS (generally recognized as safe) status (40).

In conclusion, because *L. cellobiosus* D37 shows desirable probiotic properties such as acid and bile tolerance, and anti-cancer and antibacterial activities, we suggest that it is suitable as a probiotic bacteria and can be used in the production of functional foods and the medical supplies industry. In the future, in order to completely reveal the potential of *L. cellobiosus* D37 as a probiotic, studies on reducing blood cholesterol levels, alleviating lactose intolerance, and enhancing immune function are necessary.

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