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Full Length Research Paper

Isolation and functional study of potentially probiotic *Lactobacilli* from Taiwan traditional paocai

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The objective of this study was to isolate, characterize and identify lactic acid bacteria (LAB) from Taiwan traditional pickled paocai. A total of 207 acid-producing strains were isolated and identified as catalase negative, Gram positive, with rod or bead shape and immobility by microbial and biochemical tests. None of the assayed strains showed haemolytic and gelatinase activity. Among the isolated LAB, eleven strains were able to survive at pH 2.0 and 0.3% bile salts for 3 h, utilize non-digestible oligosaccharides, show antimicrobial activity against potentially pathogenic bacteria (*Bacillus cereus*, *Micrococcus luteus*, *Salmonella typhimurium* and *Staphylococcus aureus*), and modulate cytokine production (IL-12 and IL-10) by RAW264.7 macrophage cell. Through 16S rRNA PCR product analysis and API CHL 50 kits analysis, five *Lactobacillus plantarum* (E1, E38, E40, E51 and E55), five *Lactobacillus casei* (E7, E15, E30, E33 and E40), and one *Lactobacillus rhamnosus* (E8) were identified. *L. plantarum* E51 showed the highest adherence ability and *L. casei* E33 showed an immunomodulatory effect triggering a proinflammatory type response. The results show their potential probiotic applications in health food industry.

Key words: Screening, fermented vegetables, paocai, probiotic, adhesion, cytokine.

INTRODUCTION

Probiotics are live, non-pathogenic bacterial preparations that beneficially exert health effects on their host when ingested in adequate amounts (WGO, 2008). Various lactic acid bacteria (LAB), particularly strains of *Lactobacillus* and *Bifidobacterium*, have been extensively used as probiotics. LAB are a group of Gram positive, non-spore forming, catalase negative cocci or rods which produce lactic acid as major end product from fermentation of carbohydrate. They are generally recognized as safety (GRAS) microorganisms and numbers of products containing LAB have been shown to have healthy functions (Sanders et al., 2010). These healthy functions of probiotic are: modulate gut flora, reduce gastrointestinal syndrome, reduce diarrhea disease, soften an irritable bowel, enhance the immune system, anti-food allergy, immunomodulatory activity, antimutagenic/anticarcinogenic activity and cholesterol lowering effect (Nagpal et al., 2012).

Besides, to provide health benefits, probiotics must overcome extremely low pH and the detergent effect of bile salts, and arrive at the site of action in a viable physiological state (Daniel et al., 2006; Liong and shah, 2005; Perea Velez et al., 2007). In addition, adhering to human intestinal mucosa, temporary colonization of the human gastrointestinal tract and production of antimicrobial substances or inhibition of pathogen bacteria are also the characteristics of probiotics. These characteristics may serve as suitable index for screening a probiotic from the environment.

Fermentation is one of the oldest and healthiest methods of food preservation around the world. Fermented vegetables are vegetables processed and preserved by the action of microorganisms. Many varieties of pickled and fermented foods are classified by ingredients and methods of preparation. The four general

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classes are: brined or fermented, fresh pack or quick process, fruit and relishes. Many pickled foods are fermented as part of the pickling process, but many are simply processed with brine or vinegar. During fermentation, the indigenous beneficial bacteria perform a fermentation process in which vegetables develop a pleasantly sour taste and remain rich in vitamins. Lactic acid fermentation is the best method of preservation that retains all the natural plant ingredients while improving the quality, taste and aroma. In Taiwan, many fermented (or pickled) vegetables such as fu-cai (fermented cabbage), suan-cai (pickled cabbage) and paocai (acidified cabbage, carrot, cucumber, radish and bamboo shoot) are home made popular side dishes. Paocai is a type of pickle, varies in terms of taste and method of preparation in different areas. Taiwanese paocai has crunchy texture and tangy taste, which is made with many kinds of vegetables, spices and other ingredients by anaerobic fermentation in a special container. Paocai fermentation is initiated by various microorganisms presented in the raw materials, and LAB become the dominate bacterial finally.

LAB is considered as the major contributor in fermented vegetables. A number of studies have focused on isolation of probiotic potential LAB from different resources of fermented foods. The isolation and screening of LAB from natural sources have been one of the powerful means to obtain strains for the food industry. To comprehend the emerging requirement of probiotics used in the health food industry, many studies focused on isolating the probiotic potential LAB from different resources of fermented foods (Kalui et al., 2009; Kaboosi, 2011; Kuman et al., 2012; Lavanya et al., 2011; Singh et al., 2012; Wang et al., 2010). In this study, we surveyed LAB from Taiwanese most popular consumed pickled paocai in the local market of Taipei. A specific select medium, Man-Rogosa-Sharpe (Difco^{TM} Lactobacilli MRS broth; Sparks, MD) medium containing 1.0% calcium carbonate (MRS-$CaCO_3$) was used for screening strong acid-producing LAB strain. The isolated LAB strains were identified with microbial and biochemical tests, safety identification, and assayed for the ability to survive at low pH and high bile-salt concentration. In addition, properties of adhesion, oligosaccharide-digestibility, antimicrobial activity and immune modulation ability were studied, also.

MATERIALS AND METHODS

Selection and identification of LAB

The commercialized paocai were bought from Taipei local market and chopped into small pieces. Lactobacilli MRS medium was used for isolation of the lactic acid bacteria. One gram of the homogenized sample was mixed in 9 ml sterile saline (0.3%, w/v) and stirred thoroughly. After serial dilution, 1.0 ml of each dilution was spread on the MRS medium and incubated at 37°C for 24 h. To distinguish the acid-producing bacteria from other bacteria, 1% $CaCO_3$ was added to the MRS agar. The colonies with a clear zone around them were selected for further study. The isolated LAB colonies were purified and identified by biochemical and microbial tests. The species of promising probiotic LAB strains were identified by API CHL 50 system (BioMerieux, Marcy l'Etoile, France) and 16S rRNA multiplex PCR analysis. The multiplex PCR assay was described by Kwon et al. (2004) and performed with a Personal Cycler (Biometra, Germany) by using *Taq* DNA polymerase. The PCR products were detected by electrophoresis on 1.5% agarose gel, stained with ethidium bromide.

Acid and bile salts tolerance

Acid tolerance assay was performed as previously described (Conway et al., 1987; Corcoran et al., 2005) with minor modifications. Cultures of the isolated bacteria were grown overnight in 15 ml MRS medium. The cultures were subsequently centrifuged at 1200 xg for 5 min, and washed once in an equal volume of cold PBS. The pellets were resuspended in 5 mL PBS, and the volume equivalent to approximately 10^7-10^8 CFU/mL was further centrifuged and resuspended in the appropriate volume of either two different PBS buffers (pH 2.0 and 3.0). The suspensions were incubated at 37°C in a water bath shaking with 80 rpm for 3 h. After incubation, viable bacteria counts were determined by a serial dilution on MRS plate at 37°C for 24 h. Effect of bile salts on the growth of LAB cells were shown as previously described (Daniel et al., 2006) with minor modification. Bile acids tolerance was tested by inoculation of fresh cultures into MRS broth enriched with three different Oxgall concentrations (0.2, 0.3 and 0.4%, w/v) and incubated at 37°C for 12 h. Growth curves were plotted and bile salts tolerance was determined with percentage of growth rate in bile salts. After acid (pH 2.0 and 3.0) pre-treatment, the survival LAB cells were collected by centrifugation (1200 xg, 5 min) and 0.1 M PBS (pH 7.4) was washed. Cell pellet was suspended in 3 mL MRS with 0.3% w/v Oxgall (Sigma; St. Louis, Mo.) and incubated at 37°C for 12 h. Bile salts tolerance of the LAB was determined by comparing amount of the viable LAB on MRS plate. Each assay was performed in triplicate.

Gelatinase activity

Gelatinase activity was investigated as described by Harrigan and McCance (1990). A 16 h old culture was streaked into nutrient gelatin agar (Oxoid). The plates were incubated anaerobically in anaerobic jars containing wet sachets (Merck) for 48 h at 37°C after which they were flooded with $HgCl_2$ solution (15% $HgCl_2$ in 20% HCl, v/v) and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis).

Haemolytic activity

Haemolytic activity was investigated as described by Gerhardt et al. (1981). A 16 h old culture was streaked into sterile blood agar. The blood agar was prepared by adding 7% oxblood, that had been preserved in ethylenediaminetetraacetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48 h after which they were observed for β and α haemolysis indicated by a clear zone around colonies with a greenish color for β-haemolysis but none for α-haemolysis.

Adhesion assay

Caco-2 cell (bought from the cell bank of BCRC, Taiwan) was cultured in MEM (Modified Eagle's Medium) medium with

nonessential amino acids (Mediatech), 10% fetal bovine serum (Sigma), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma) and cultured at 37°C, 5% CO_2 and 95% air for 14 days to use as adhesion model. The adhesion assay and the numbers of adhered LAB cells per Caco-2 cell was determined by counting LAB cells on Caco-2 cell, in 20 randomly selected microscopic fields as described by Jacobsen et al. (1999).

Antibacterial activity assay

The antibacterial activity was tested with disc diffusion method (Tagg and McGiven, 1971). Pathogenic indicator bacteria include *Staphylococcus aureus* BCRC10451, *Bacilus cereus* BCRC10603, *Micrococcus luteus* BCRC10449, and *Salmonella typhimuriums* BCRC10474. Spent culture supernatant (50 µL) with or without neutralization were dropped in sterile paper disc (8 mm in diameter). The agar plates were incubated at 37°C overnight and the diameters of the inhibition zones on the agar plate were measured. A clear zone of more than 10 mm around paper disc was scored as positive. Each test was performed in duplicate.

Oligosaccharides digestible strain assay

Oligosaccharides digestible stain assay was tested with oligosaccharides-MRS method (Kaplan and Hutkins, 2000). An oligosaccharides-MRS agar medium was prepared by adding 2% (wt/vol) purified fructooligosaccharides (FOS), isomaltooligosaccharides (IMOS) or gentiooligosaccharides (GOS) to MRS agar containing 0.05% L-cysteine, 1.5% agar and 30 mg bromcresol purple per liter. The MRS basal medium (MRS without carbohydrate) was autoclaved, and the oligosaccharides were filter sterilized and then added to the tempered agar. The positive strain was also confirmed with cell density in MRS basal broth containing either 2% glucose or 2% oligosaccharides.

Effect of isolated LAB on macrophage induced production of cytokines

Mouse macrophage cell line, RAW264.7 (ATCC TIB-71), was cultured in DMEM (Dulbecco's Modified Eagle's Medium) medium with 10% FETALCLONE® III, and cultured at 37°C, 5% CO_2 and 95% air for 2 to 3 days. Macrophage cell was seeded at 1×10^5 cell/mL into 96-well culture plates and was incubated for 4 h; then, the medium was changed and the macrophage cells were cultures with 200 µL additional stimuli, such as heat-killed lactobacilli suspensions and *Lactobacillus casei* Shirota (LcS) as control, for 24 h. The cell density of *Lactobacillus* strains were adjusted to OD600 nm to 1.0 and killed at 100°C for 30 min (Haller et al., 2002). The absence of viable cells was confirmed by a standard plate count as mentioned above. Heat-killed lactobacilli was collected by centrifugation (1200 xg, 5 min) and dissolved in DMEM medium. The heat-killed lactobacilli suspensions were added to the well at 1×10^9 CFU/well. After incubation, the supernatants were harvested and stored at -80°C until cytokines analysis. Cytokines (IL-12 and IL-10) were determined with Pierce® Mouse IL-12 and IL-10 ELISA kit (Pierce Co. Rockford, IL).

RESULTS AND DISCUSSION

Counts of LAB

To comprehend the emerging requirement of probiotics used in the health food industry, the isolation and screening of LAB from natural sources have been one of the powerful means to obtain strains for food industry. Five commercialized fermented vegetables of different makers were bought and prepared for their LAB analysis. Total bacterial count was determined with plate count agar (PCA) and MRS medium. The former was used for determining total microbial counts and the latter was used for measuring total LAB counts. The total LAB counts of the five brands of fermented paocai were similar to that of the total microbial counts. This means that LAB is the major contributor of the pickled paocai. The pickled paocai are the popular side dish in Taiwan. Because the pickled vegetables contained larger amount of LAB; therefore, one can get much LAB from eating pickled vegetables, taking the beneficial bacteria to our intestine to keep our digestive system regularly healthy. Screening for the high acid-producing strains, 207 colonies with high acid producing ability from MRS-$CaCO_3$ medium were selected and identified as catalase negative, Gram positive, with rod or bead shape and immobility by biochemical and microbial tests. They were preliminarily identified as LAB.

Acid tolerance and bile salt tolerance

To study the probiotic properties, the following tests were performed: test for acid-tolerance (pH 2.0 or 3.0) and bile salt-tolerance (0.2, 0.3 and 0.4% w/v bile salts). The bile salt in animal intestine is an important factor which affects the LAB viability. Although, the bile salt concentration of the human gastrointestinal GI tract varies, most studies used 0.3% w/v bile salt as one substitute for human bile salt (Lin et al., 2006; Liong and Shah, 2005; Sahadeva et al., 2011). The results showed that 33 of the 207 colonies could survive with 0.3% bile salt-treatment. Then, 11 of the 33 colonies could endure acid damage and showed little or no decrease in viable cell numbers even after 3 h incubation at pH 3.0 as compared to that of the commercialized probiotic strain LGG (Table 1). These promising probiotic LAB strains were identified in the species with API 50 CHL kit and 16S rRNA multiplex PCR product analysis (Figure 1). Five strains (E1, E38, E46, E51 and E55) were identified as *L. plantarum*. Five strains (E7, E15, E30, E33 and E40) were identified as *L. casei*. One strain (E8) was identified as *L. rhamnosus*.

In another approach, the ability of the eleven selected strains to propagate, rather than just survive low pH and high bile salt conditions, was determined. Eleven strains were pre-incubated in 0.1 M PBS at pH 2.0 or 3.0 for 3 h before resuspending in MRS broth with 0.3% w/v bile salts. After cultivation, the growth of LAB was observed and numbers of flora were counted (Table 1). The survival rate was better at pH 3.0 to 0.3% bile salt than at pH 2.0 to 0.3% bile salt. Conway et al. (1987) reported that probiotic strains are likely to be buffered by food and

Table 1. Survival of the 11 isolated LAB under acid, bile salts and gastrointestinal tract simulation treatment.

Isolated strain*	Acid treatment (CFU/ml)**			Bile salts treatment (%)***			Gastrointestinal tract (CFU/ml)****	
	0 h	3 h		0.2%	0.3%	0.4%	pH 2.0,	pH 3.0,
	pH 7.4	pH 2.0	pH 3.0	bile salt	bile salt	bile salt	0.3% bile salt	0.3% bile salt
LGG	$4.5\pm0.71\times10^{7}$	30±2.83	$3.0\pm0.14\times10^{5}$	37.86	-	-		
E1 (*Lactobacillus plantarum*)	$1.2\pm0.28\times10^{8}$	$1.4\pm0.14\times10^{3}$	$3.0\pm0.71\times10^{5}$	63	51	39	$3.7\pm1.27\times10^{4}$	$1.5\pm0.42\times10^{7}$
E7 (*Lactobacillus casei*)	$1.1\pm0.42\times10^{8}$	$5.7\pm1.13\times10^{3}$	$1.0\pm0.14\times10^{6}$	70	51	52	$4.9\pm0.71\times10^{3}$	$8.3\pm1.98\times10^{6}$
E8 (*Lactobacillus rhamnosus*)	$3.3\pm1.13\times10^{7}$	$1.1\pm0.42\times10^{3}$	$1.0\pm0.28\times10^{6}$	63	51	52	$1.8\pm0.57\times10^{4}$	$1.1\pm0.57\times10^{5}$
E15 (*Lactobacillus casei*)	$1.5\pm0.42\times10^{8}$	$2.4\pm0.71\times10^{3}$	$4.2\pm0.85\times10^{7}$	77	55	35	$1.1\pm0.14\times10^{3}$	$1.2\pm0.42\times10^{7}$
E30 (*Lactobacillus casei*)	$1.8\pm0.28\times10^{8}$	$1.3\pm0.42\times10^{4}$	$1.5\pm0.14\times10^{7}$	100	100	94	$4.7\pm0.99\times10^{4}$	$1.9\pm0.42\times10^{6}$
E33 (*Lactobacillus casei*)	$3.3\pm0.99\times10^{8}$	$1.4\pm0.71\times10^{4}$	$3.1\pm0.57\times10^{7}$	67	73	67	$4.1\pm0.71\times10^{4}$	$2.7\pm0.14\times10^{6}$
E38 (*Lactobacillus plantarum*)	$1.4\pm0.35\times10^{7}$	$1.3\pm0.28\times10^{3}$	$3.0\pm0.42\times10^{6}$	100	100	100	$3.4\pm0.71\times10^{4}$	$1.4\pm0.14\times10^{6}$
E40 (*Lactobacillus caei*)	$1.7\pm0.14\times10^{8}$	$3.0\pm0.99\times10^{3}$	$3.0\pm0.57\times10^{5}$	78	70	60	$3.7\pm0.57\times10^{4}$	$1.4\pm0.42\times10^{7}$
E46 (*Lactobacillus plantarum*)	$3.3\pm0.71\times10^{8}$	$2.7\pm0.42\times10^{2}$	$1.5\pm0.28\times10^{8}$	100	83	74	$1.0\pm0.28\times10^{4}$	$2.0\pm0.99\times10^{8}$
E51 (*Lactobacillus plantarum*)	$9.8\pm1.13\times10^{8}$	$1.4\pm0.14\times10^{4}$	$6.8\pm1.84\times10^{7}$	100	100	100	$1.0\pm0.42\times10^{4}$	$5.0\pm1.84\times10^{7}$
E55 (*Lactobacillus plantarum*)	$1.5\pm0.28\times10^{8}$	$2.4\pm0.57\times10^{3}$	$1.2\pm0.42\times10^{7}$	100	100	100	$2.3\pm0.57\times10^{4}$	$2.8\pm0.71\times10^{6}$

*: *Lactobacillus* GG is probiotic strain and used as control in this study. Isolated *Lactobacillus* was identified in the species with API CHL50 system and 16S rRNA multiplex PCR analysis; **: Bacterial count of each sample was obtained by average of three tests; ***: Percentage of growth rate in bile salts (bile salts tolerance) = (Increment of OD in MRS broth with 0.2, 0.3 or 0.4% bile salts)/ increment of OD in MRS broth without bile salts) × 100. ****: The survival bacteria number of treatment with different low pH PBS buffer for 3 h and growth in MRS broth with 0.3% bile salts for 12 h.

other carrier matrix molecules following consumption and not to be exposed to low pH in the stomach. Although, partial strains had lower acid tolerance at pH 2.0, the surviving of LAB was increased with food or feed. The results indicated that these strains will not only survive at the low pH of the stomach but may be able to grow in higher bile salts concentration environment of the intestine.

Adhesive ability and antimicrobial activity

Adhesive ability of the eleven strains was simulated by adhering to the monolayer of Caco-2 cells. Considerable variation among the bacteria was observed (Table 2). One strain E51 showed highly adhesive ability, two strains E33 and E38 showed moderate-to-low adhesive ability, and others showed little adhesion. The ability of LAB to adhere to mucosal surfaces is important for bacterial to inhabit the human gastrointestinal tract, inhibit the cell association and cell invasion by bacterial pathogens (Jacobsen et al., 1999). In this regard, only a few LAB strains conform to this probiotic function.

The inhibitory activities of the LAB isolates in the form of cell free spent broth tested against common food pathogens are shown in Table 3. All the 11 isolated strains showed inhibitory activities against the indicator bacteria, *S. aureus*, *B. cereus*, *Shigella flexneri* and *S. typhimuriums*. The inhibition effects are considered to result from the acids produced in the culture filtrate or might result from bacteriocin-like substances produced by the LAB (Servin, 2004). The actual inhibition effects should be studied in the future.

Oligosaccharide utilization

All tested strains were sufficiently rich to promote colony formation in basal MRS broth, and could metabolize IMOS, GOS and FOS to produce enough acid to cause a noticeable color change of the selection medium. However, only 3 strains (E15, E30 and E55) could hydrolyze FOS (Table 3). To compare the utilization of oligosaccharides by the isolated LAB, two strains *L. casei* E30 (could hydrolyzed FOS) and *L. plantarum* E51 (could not hydrolyze FOS) were used. The utilization ability was evaluated by the growth of bacteria and tracing the hydrolysates by HPLC. The growth density on oligosaccharides as

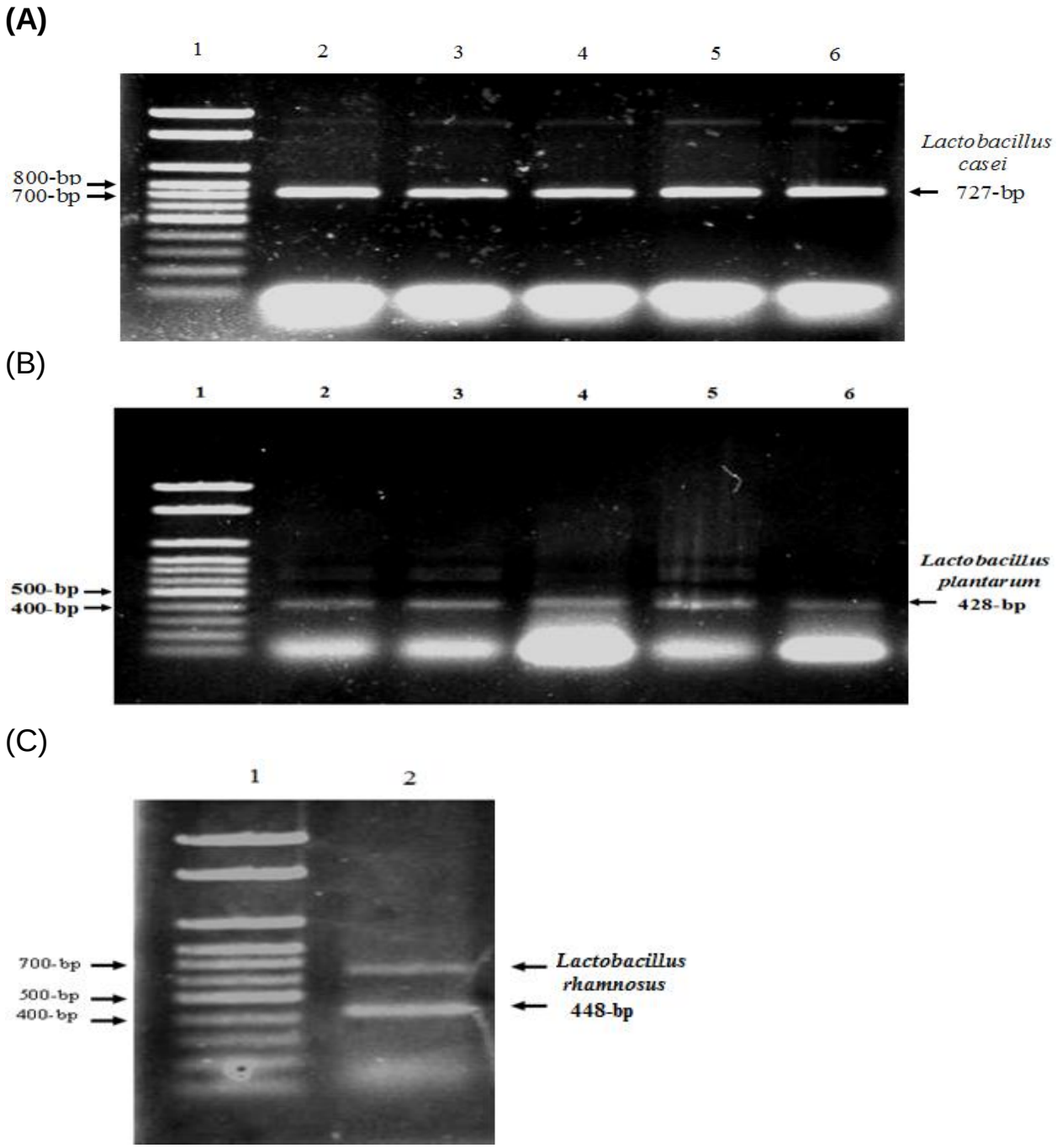

Figure 1. The results of species analysis on probiotics LAB. (A) Identified as *Lb. casei* strain. Lane 1: 1-kb DNA Ladder marker; Lane 2: E7; Lane 3: E15; Lane 4: E30; Lane 5: E33; Lane 6: E40. (B) Identified as *Lb. plantarum* strain. Lane 1: 1-kb DNA Ladder marker; Lane 2: E1; Lane 3: E38; Lane 4: E46; Lane 5: E51; Lane 6: E55. (C) Identified as *Lb. rhamnosus* strain. Lane 1: 1-kb DNA Ladder marker; Lane 2: E8.

compared to sugar-free broth is shown in Figure 2. The *L. casei* E30 strain could metabolize FOS, IMOS and GOS. It had higher growth response as compared to sugar-free broth. On the contrary, *L. plantarum* E51 strain could not use FOS. The bacterial growth response range was less than IMOS and GOS, remarkably.

Cytokine expression ability of macrophage induced with LAB

To analyze the effect of the isolated LAB strains on macrophage cytokine production, macrophage cell line RAW264.1 were cultured with heat-killed LAB for 24 h,

Table 2. Numbers of the 11 isolated LAB adhered to Caco-2 cell monolayer.

Isolated strain	LAB adherence Index (CFU/cell)**
LGG*	45 (+)
E1 (*Lactobacillus plantarum*)	5 (-)
E7 (*Lactobacillus casei*)	25 (+)
E8 (*Lactobacillus rhamnosus*)	0 (-)
E15 (*Lactobacillus casei*)	38 (+)
E30 (*Lactobacillus casei*)	8 (-)
E33 (*Lactobacillus casei*)	47 (+)
E38 (*Lactobacillus plantarum*)	78 (+)
E40 (*Lactobacillus caei*)	3 (-)
E46 (*Lactobacillus plantarum*)	31 (+)
E51 (*Lactobacillus plantarum*)	238 (++)
E55 (*Lactobacillus plantarum*)	10 (-)

*: LGG is probiotic strain and used as control in this study. **: Expressed as the average number of LAB adhering to Caco-2 cells, adherence index is carried out on 20 randomized microscopic fields. The number of LAB adhering to Caco-2 cells, when lower than 15 CFU/cells is seen as negative adhesion of LAB and shown as - ; 15 to 100 CFU/cell is seen as positive adhesion and shown as+; number higher than 100 CFU/cell is stronger and shown as ++.

Table 3. Inhibitory activity of spent culture supernatants of the 11 isolated LAB for food bone pathogenic bacteria.

Strain no.	Gram positive strain			Gram negative strain
	S. aureus **BCRC10451**	***B. cereus*** **BCRC10603**	***M. luteus*** **BCRC10449**	***S. typhimurium*** **BCRC10474**
Lactobacillus plantarum E1	+*	++	+	+
Lactobacillus casei E7	+	++	+	+
Lactobacillus rhamnosus E8	+	++	+	+
Lactobacillus casei E15	+	++	+	+
Lactobacillus casei E30	+	++	+	+
Lactobacillus casei E33	+	++	+	+
Lactobacillus plantarum E38	+	++	+	+
Lactobacillus casei E40	+	++	+	+
Lactobacillus plantarum E46	+	+	+	+
Lactobacillus plantarum E51	+	+	+	+
Lactobacillus plantarum E55	+	++	+	+

*: "-" shown as diameter of inhibition zone, ≤10 mm; "+" shown as diameter of inhibition zone, 11 to 14 mm; "++" shown as diameter of inhibition zone, 15 to 19 mm; "+++" shown as diameter of inhibition zone, ≥20 mm.

and IL-10 and IL-12 concentrations in the supernatant were measured by ELISA. All the tested LAB strains could induce macrophage to produce cytokine IL-10 and IL-12 in different amount. Variations in IL-10 concentrations were substantial with values ranging from 350 to 4000 pg/mL, depending on the LAB strains (Figure 4). Strains E55, E1 and E40 produced higher level of IL-10 than the other tested strains including the standard strain *L. casei* Shirota (LcS). For IL-12, we also observed significant variations between strains, covering a range of cytokine levels of 70 to 300 pg/mL (Figure 3). Strain E 33, E 40 and E 30 induced highest level of IL-12 among the tested LAB including the standard LcS and LGG, significantly ($P<0.05$). This result was similar to that of Kimoto et al. (2004). It is revealed that cell wall components, which constitute heat-killed cells, may be a factor for immune modulation.

In intestine, when the LAB is in contact with the immunocytes of Peyer's patches, the TLRs (toll-like receptor) will trigger the host cells to express and

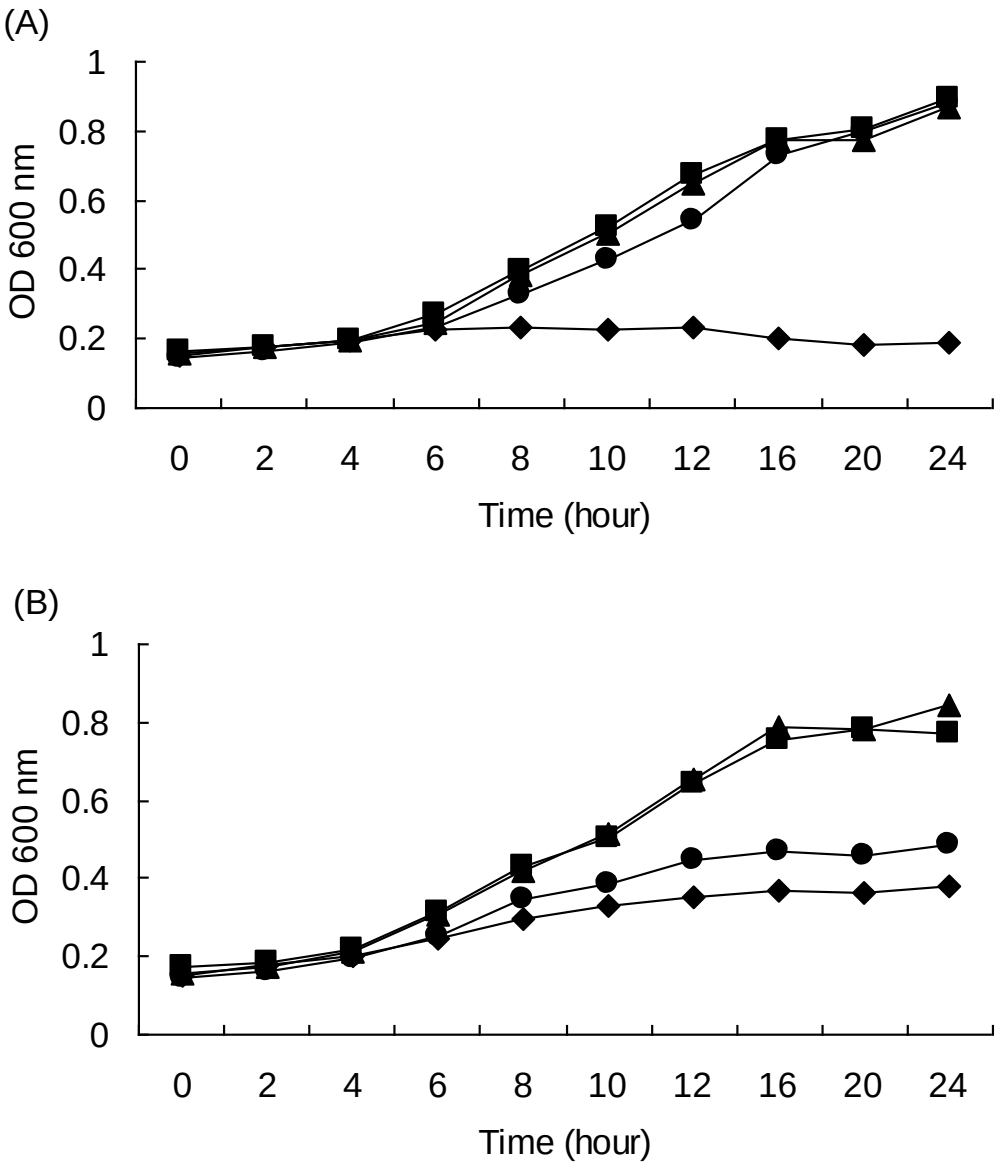

Figure 2. Growth analysis of *L. plantarum* E51 (A) and *L. casei* E30 (B) in MRS broth containing oligosaccharides. The broth which contains free sugar is shown as◆, 2% GOS is shown as ■, 2% IMOS is shown as ▲, and 2% FOS is shown as ●.

produce different kinds of cytokines, chemokines and inflammatory effector molecules. They also induce up-regulation of costimulatory molecules on dendritic cell (DC) and regulate the polarization of CD4 T helper (Th) lymphocytes into Th1, Th2 or Th3/Tr1 subsets. Cytokines function as a network for communication between immunocytes controlling immune responses or tolerance. IL-12 is an important pro-inflammatory cytokine to promote development of Th1 cells, augment NK cell function, increase IFN-γ producing capacity and suppress the overpression of Th2 phenotype cells (Cross et al., 2001). On the other hand, IL-10 was an anti-inflammatory cytokine which when secreted by Th2 phenotype cells, reduces secretion of Th1 cytokines, suppresses macrophage activation, and possibly even influences cytotoxic T and NK cells. Based on our data, E33 produced higher level of IL-12 and lower concentration of IL-10 than other tested strains, this strain belongs to the immunostimulatory probiotics, which can promote activation of natural killer cells and the development of Th1 cells and leading to inflammatory response. On the contrary, E55 could produce higher level of IL-10 and lower level of IL-12 than other tested strains, this strain can be categorized as immunoregulatory probiotics, which can promote the development of Treg cells and control inflammatory response.

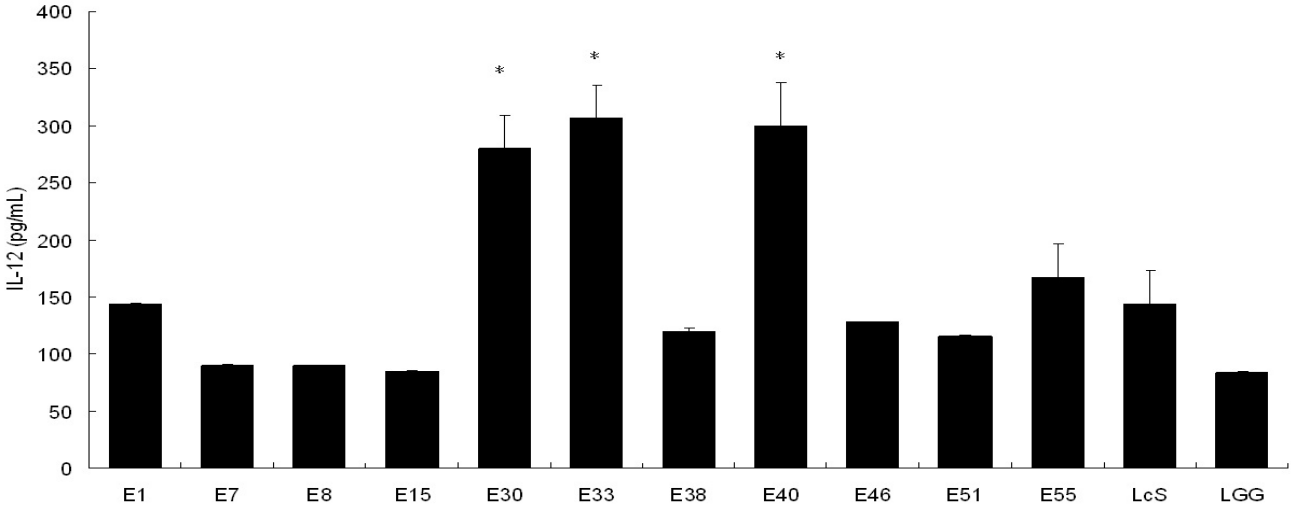

Figure 3. Production of IL-12 by macrophage RAW 264.1 induced by heat-killed LAB suspensions. Production of cytokines in culture supernatant was detected by ELISA. LGG *(Lactobacillus rhamnosus* GG) and LcS (*Lactobacillus casei* Shirota) are probiotic strains and used as positive control.

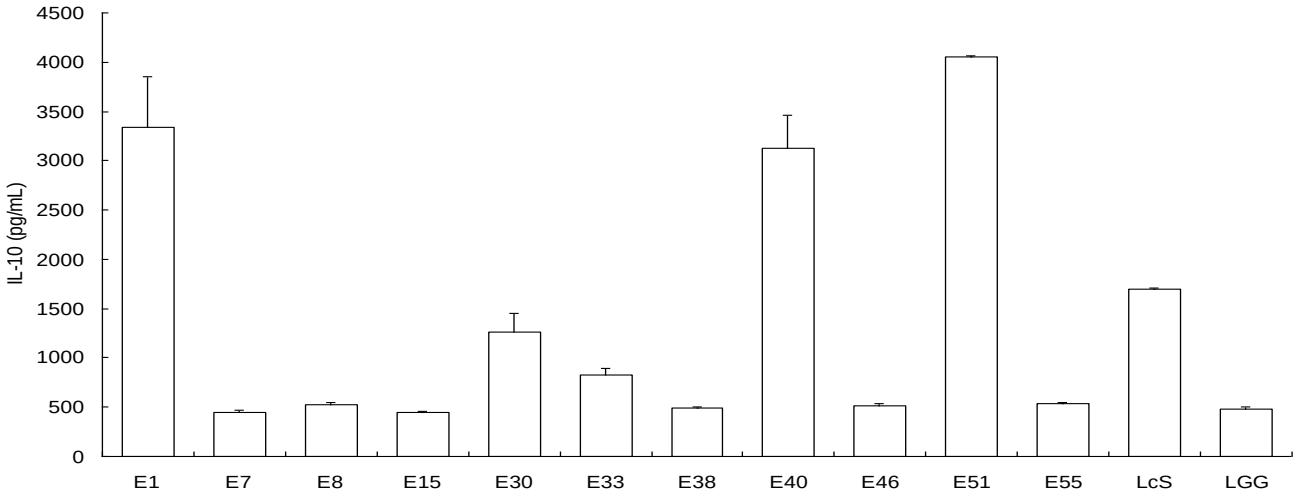

Figure 4. Production of IL-10 by macrophage RAW 264.1 induced by heat-killed LAB suspensions. Production of cytokines in culture supernatant was detected by ELISA. LGG (*Lactobacillus rhamnosus* GG) and LcS *(Lactobacillus casei* Shirota) are probiotic strains and used as positive control.

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