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ARTICLE

Screening and Characterization of Lactic Acid Bacteria Strains with Anti-inflammatory Activities through *in vitro* and *Caenorhabditis elegans* Model Testing

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Abstract

The present study was conducted to screen candidate probiotic strains for anti-inflammatory activity. Initially, a nitric oxide (NO) assay was used to test selected candidate probiotic strains for anti-inflammatory activity in cultures of the murine macrophage cell line, RAW 264.7. Then, the *in vitro* probiotic properties of the strains, including bile tolerance, acid resistance, and growth in skim milk media, were investigated. We also performed an *in vitro* hydrophobicity test and an intestinal adhesion assay using *Caenorhabditis elegans* as a surrogate *in vivo* model. From our screening, we obtained 4 probiotic candidate lactic acid bacteria (LAB) strains based on their anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated RAW 264.7 cell cultures and the results of the *in vitro* and *in vivo* probiotic property assessments. Molecular characterization using 16S rDNA sequencing analysis identified the 4 LAB strains as *Lactobacillus plantarum*. The selected *L. plantarum* strains (CAU1054, CAU1055, CAU1064, and CAU1106) were found to possess desirable *in vitro* and *in vivo* probiotic properties, and these strains are good candidates for further investigations in animal models and human clinical studies to elucidate the mechanisms underlying their anti-inflammatory activities.

Key words: lactic acid bacteria, probiotics, anti-inflammatory, *Caenorhabditis elegans*

Introduction

Lactic acid bacteria (LAB) are gram-positive organisms that are found throughout nature, in vegetables, meats, dairy products, and many parts of human body, including the gastrointestinal (GI) tract. Probiotics can be defined as "live microbial food ingredients that, when administered in adequate amounts, confer a health benefit on a host" (FAO/WHO, 2001). These beneficial effects include amelioration of lactose intolerance, anti-mutagenic and anti-carcinogenic activities, reduction of serum cholesterol, modulation of immune responses, and anti-inflammatory activities (Maldonado *et al*., 2007; Mishra and Prasad, 2005). The genera most commonly used in probiotic applications are *Lactobacillus*, *Pediococcus*, *Weissella*, *Streptococcus*, and *Bifidobacterium*.

Macrophages are tissue-based phagocytes derived from monocytes that play a central role in the initiation of first line immune defense (Chon *et al*., 2009). Lipopolysaccharide (LPS) of gram-negative bacteria and lipoteichoic acid (LTA) of gram-positive bacteria can induce macrophage activation. Activated macrophages induce immune responses. Nitric oxide (NO) is generated via oxidation of L-arginine to L-citrulline by NO synthase (NOS) (Lee *et al.*, 2010). During an inflammatory response, activated macrophages secrete large amounts of NO via inducible NOS (iNOS) stimulated by inflammatory signals such as pro-inflammatory cytokines and endotoxins (LPS) from several cell types (Korhonen *et al.*, 2002). It has been reported that NO synthesized in activated macrophages can function as a mediator of numerous tumoricidal and antimicrobial effects (Lorsbach *et al.*, 1993).

Recently, numerous studies have focused on modulating the composition of the intestinal microbiota and probiotic LAB strain-mediated inhibition of inflammatory

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responses. *In vitro* studies using murine RAW 264.7 macrophages and human HT-29 cells have shown that *Lactobacillus plantarum* KFCC11389P (Chon *et al*., 2009), *L. rhamnosus* GG (Lee *et al*., 2012), *L. casei* MCL (Choi *et al*., 2012), and *L. casei* LC-EAS (Karthikeyan *et al*., 2013) have immunomodulatory effects. In addition, based on the results of studies in an *in vivo* colitis mouse model, anti-inflammatory effects have been reported for *L. salivarius* 433118 (Feighery *et al*., 2008), *L. plantarum* HY115, *L. brevis* HY7401 (Lee *et al*., 2008), and *L. plantarum* Lp91 (Duary *et al*., 2011).

Two of the most important selection criteria for potential probiotic candidate strains are the ability to adhere to the intestinal epithelial surface and colonize the gastrointestinal (GI) tract (Byrd and Bresalier, 2004; Ouwehand and Salminen, 2003; van Tassell and Miller, 2011). Many studies have focused on alternative *in vitro* models to screen probiotic strains for these abilities, including using propagated human intestinal cells, such as HT-29 and Caco-2 cells, and mucin-adhesion assays (Carasi *et al*., 2014; Duary *et al*., 2011; Valeriano *et al*., 2014). Recently, a *Caenorhabditis elegans* surrogate *in vivo* model has been successfully used for simple, rapid, and economic high-throughput screening of potential probiotic bacteria (Park *et al*., 2014). The practical advantages of using this model system for screening are that the body of *C. elegans* is transparent, which allows clear observation of all cells in mature animals and at all developmental stages (Leung *et al*., 2008) and that its intestinal cells are similar in structure to those in humans (McGhee, 2007).

In this study, we investigated the ability of selected probiotic candidate strains to mediate anti-inflammatory activities in cultures of murine RAW 264.7 macrophages. Selected strains exhibiting high levels of NO production inhibition activity in LPS-stimulated RAW macrophages were identified, and their probiotic properties were further characterized using *in vitro* assays and a *C. elegans* model. Finally, we studied the milk-fermenting ability of the selected strains to assess their suitability as probiotic strains for functional fermented milk production.

Materials and Methods

Selection of strains from healthy adult feces

As an appraisal for the application of new probiotic cultures, many strains were isolated from healthy human feces using Rogosa SL agar as a selective medium (Choi *et al*., 2012). Typical colonies on the agar plates were selected randomly and subcultured in MRS broth, and pure cultures were obtained by restreaking on MRS agar. Basic characterization studies, such as Gram staining and catalase tests, were performed. All isolated LAB strains were maintained in MRS broth containing 25% glycerol at -80 °C until use.

Isolate identification

The bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using universal primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGYTACCTTGTTACGACTT-3) in a LifeTouch thermal cycler (Alpha Laboratories, UK). The cycling program was as follows: denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 47°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. The PCR products were purified with a PCR purification kit (Qiagen, USA) and sequenced by Solgent (Korea).

Macrophage cell culture

RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin and penicillin at 37°C in a humidified 5% CO_2 incubator. Cell numbers and viability were measured by using Trypan blue (Amresco, USA) and a hemocytometer. Confluent cells were subcultured every 2 d as described previously (Choi *et al*., 2012).

Nitric oxide assay

RAW 264.7 cells were placed into 24-well plates at a density of 1.0×10^5 cells/well. LPS (100 ng/mL) and LAB fractions (at 2.5% final concentration, 10 times concentrated) were added. After 24 h, the concentration of NO was determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagent (Sigma, USA) as previously described (Green *et al*., 1982). An aliquot of the culture supernatant (50 μL) was mixed with an equal volume of Griess reagent and incubated for 15 min at room temperature. The absorbance at 540 nm was measured using a microplate reader. The nitrite concentration was determined based on a standard curve prepared using sodium nitrite (Kim *et al.*, 2007).

Acid tolerance

As described previously (Hedin *et al*., 2000), bacterial cultures (initial density, 10^7 CFU/mL) were incubated in MRS broth overnight at 37°C. The overnight cultures were divided into two equal aliquots. To measure acid tol-

erance, bacterial cells harvested from one aliquot were suspended in MRS broth containing 0.05% L-cystein·HCl adjusted to pH 3.0 (by adding 4 M HCl) and incubated for 3 h at 37°C. Cells harvested from the second culture aliquot were used as a control. After incubation, the acidic culture was neutralized by adding 0.1 M PBS (pH 6.2), 10-fold serial dilutions were made. Live cells were counted on MRS agar plates after incubation for 48 h at 37°C. Acid tolerance was expressed as a percentage of the number of viable cells after incubation in MRS broth (pH 3.0) relative to that of the control and was calculated using the following equation:

Acid tolerance (%) = (Log CFU/mL after 3 h of exposure) / (initial Log CFU/mL) × 100

Bile tolerance

Bile tolerance was assessed according to the method of Gilliland *et al.* (1990). Briefly, MRS broth and MRS broth supplemented with 0.3% oxgall were inoculated with an overnight culture (0.5% initial inoculum). After 12 h of culture at 37°C, the optical density at 620 nm (OD_{620}) was measured. Bile resistance was expressed as a percentage of the number of viable cells after incubation in MRS broth with oxgall compared to that of the control (without oxgall) and was calculated using the following equation:

Bile resistance (%) = (OD_{620} in MRS broth with oxgall) / (OD_{620} in MRS broth without oxgall) × 100

Hydrophobicity

Cell surface hydrophobicity was determined using the method of Valeriano *et al.* (2014). Briefly, an overnight culture was harvested (13,000 rpm, 15 min), washed twice, and resuspended in PBS to a final OD_{600} of ~0.5, which was designated as A_0. An aliquot (3 mL) of the cell suspension was mixed with 1 mL of toluene. The mixture was mixed vigorously for 90 s and then left to stand at 37°C for 1 h to allow phase separation. After the toluene phase was removed, the OD_{600} of the aqueous phase was measured and designated as A_1. Each experiment was performed in triplicate from independent cultures. Cell surface hydrophobicity was determined as the percentage decrease in the absorbance of the aqueous phase after exposure to toluene, which was calculated using the following equation (Valeriano *et al*., 2014):

Hydrophobicity (%) = $[1 - (A_1/A_0)] \times 100$

where,

A_0 : OD_{600} value of the original suspension

A_1 : OD_{600} value of the aqueous phase after extracting with toluene

***C. elegans* intestine adhesion assay**

The *C. elegans* strain used in this study was CF512 f*er-15(b26)II;fem-1(hc17)IV* (Kim and Mylonakis, 2012). This *C. elegans* strain was routinely maintained according to the method described by Brenner (1974) using nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50. The isolated LAB strains were screened for colonization of the *C. elegans* intestinal tract according to a previously described method (Park *et al*., 2014). Briefly, after exposing *C. elegans* to individual LAB strains on NGM plates containing nystatin for 5 d, 10 worms were randomly selected, washed twice with M9 buffer, and place on brain-heart infusion (BHI) plates containing kanamycin and streptomycin. These plates then were exposed to gentamycin (5 μL of a 25 μg/mL solution) for 5 min. After the worms were washed three times with M9 buffer, they were pulverized using a pestle (Kontes Glass Inc., USA) in a 1.5-mL Eppendorf tube containing M9 buffer supplemented with 1% Triton X-100. The worm lysate was serially diluted (10-fold) in M9 buffer and plated on MRS agar (pH 5.0). After incubation for 48 h at 37°C, live bacterial cells in the lysates were counted.

Culture characteristics in skim milk

The cell viability, pH, and titratable acidity (TA) of the LAB selected strains and *L. casei* YIT 9029 (obtained from Korea Yakult Co.) were assessed during growth in skim milk media (Choi *et al*., 2012). Overnight cultures were containing 10% skim milk and 2% glucose were incubated at 37°C in a water bath. Viable cells were counted at 4 h intervals for 36 h. Cultured cells were serially diluted in PBS and then plated on MRS agar. Plates were incubated at 37°C for 48 h. The pH was measured using a pH meter (Seven easy™ S20; Mettler Toledo, USA). To measure the TA, the cultured samples were titrated with 0.1 N NaOH. TA was expressed as the percentage of lactic acid contents in the sample.

Results and Discussion

Screening and identification of isolates

Molecular characterization of 24 fecal isolates using 16S rDNA sequencing analysis (Table 1) showed that *L. plantarum* (6 strains) was the most common species, followed by *L. sakei* subsp. *sakei* (4 strains), *Pediococcus pentosaceus* (3 strains), *L. curvatus* (2 strains), *L. salivar-*

Table 1. 16S r DNA sequence homology with the type strains obtained from Ez-taxon search

Strain ID	Identified as	Type strain[a]	Homology (%)
1	*Lactobacillus plantarum* CAU1045	ATCC 14917^T	99.27
2	*Lactobacillus paracasei* subsp. *tolerans* CAU1048	JCM 1171^T	99.38
3	*Lactobacillus plantarum* CAU1054	ATCC 14917^T	99.36
4	*Lactobacillus plantarum* CAU1055	ATCC 14917^T	99.81
5	*Lactobacillus plantarum* CAU1064	ATCC 14917^T	99.14
6	*Streptococcus infantarius* subsp. *coli* CAU1085	NCDO 964^T	99.93
7	*Lactobacillus plantarum* CAU1106	ATCC 14917^T	99.68
8	*Pediococcus pentosaceus* CAU1212	DSM 20336^T	99.06
9	*Lactobacillus acidophilus* CAU1214	ATCC 4356^T	99.72
10	*Pediococcus pentosaceus* CAU1216	DSM 20336^T	99.31
11	*Weissella cibaria* CAU1221	KACC 11862^T	99.44
12	*Weissella viridescens* CAU1222	NRIC 1536^T	99.93
13	*Pediococcus pentosaceus* CAU1223	DSM 20336^T	99.32
14	*Weissella viridescens* CAU1224	NRIC 1536^T	98.39
15	*Weissella cibaria* CAU1225	KACC 11862^T	99.52
16	*Lactobacillus sakei* subsp. *sakei* CAU1241	DSM 20017^T	99.86
17	*Lactobacillus sakei* subsp. *sakei* CAU1242	DSM 20017^T	99.78
18	*Lactobacillus curvatus* CAU1243	LMG 9198^T	99.64
19	*Lactobacillus curvatus* CAU1244	LMG 9198^T	99.64
20	*Lactobacillus sakei* subsp. *sakei* CAU1245	DSM 20017^T	99.86
21	*Lactobacillus sakei* subsp. *sakei* CAU1273	DSM 20017^T	99.90
22	*Lactobacillus salivarius* CAU1301	ATCC 11741^T	99.86
23	*Lactobacillus salivarius* CAU1302	ATCC 11741^T	99.86
24	*Lactobacillus plantarum* CAU1364	ATCC 14917^T	99.81

[a]ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; JCM, Japan Collection of Microorganisms; KACC, Korean Agricultural Culture Collection; LMG, LMG Bacteria Collection Universiteit Gent; NCDO, National Collection of Dairy Organism; NRIC, NODAI Research Institute Culture Collection

ius (2 strains), *Weissella cibaria* (2 strains), *W. viridescens* (2 strains), *L. paracasei* subsp. *tolerans* (1 strain), *L. acidophilus* (1 strain), and *Streptococcus infantarius* subsp. *coli* (1 strain).

Nitric oxide assay

Initially, the anti-inflammatory activity of the LAB isolates was investigated in LPS-stimulated RAW 264.7 cells. Heat-killed whole bacterial cells of the isolated LAB strains were added to the RAW cell cultures, and inhibition of NO production was evaluated. NO is a suspected mediator in the development of diseases associated with chronic inflammation, including cancer and inflammatory bowel disease. NO also has various biological functions in many types of immune cells, including induction of the bactericidal effect in macrophages and signal transduction during inflammation (Jeong *et al*., 2010). The ability of the LAB strains to inhibit NO production was expressed as a relative NO rate (%) compared with the NO concentration in the controls (Fig. 1). The positive RAW 264.7 cell control culture was treated with LPS only, and the negative control was treated with PBS. As shown in Fig. 2, potential anti-inflammatory effects were observed in cultures with 8 isolated LAB strains (CAU1054, CAU 1055, CAU1064, CAU1106, CAU1245, CAU1273, CAU 1301, and CAU1302) belonging to the genus *Lactobacillus*. Four *L. plantarum* strains reduced NO production; however, the other 2 *L. plantarum* strains increased NO production in the RAW cell cultures, suggesting that the anti-inflammatory effects are strain specific, even within the same species. In addition, some strains of *L. acidophilus*, *L. sakei* subsp. *sakei*, and *L. salivarius* also inhibited NO production. However, other LAB strains, including *L. paracasei*, *S. infantarius* subsp. *coli, P. pentosaceus*, *W. cibaria*, *W. viridescens*, and *L. curvatus* increased NO production. According to Chon *et al.* (2009), intracellular metabolites or molecules from *L. plantarum* KFCC11389P have inhibitory effects on the production of the pro-inflammatory cytokines IL-6 and TNF-α in LPS-stimulated RAW 264.7 macrophages. However, these anti-inflammatory effects were not detected when viable or heat-killed cells were used, suggesting that the *L. plantarum* KFCC11389P cells did not affect the anti-inflammatory cytokines, and thus could not exert the immune-

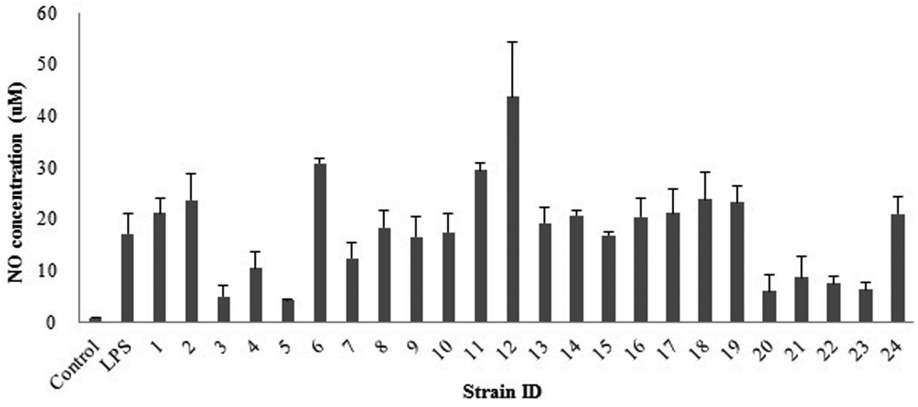

Fig. 1. Nitric oxide concentration (μM) in the RAW 264.7 cells treated with the heat killed whole cells of 24 LAB isolates (Strain ID: See the Table 1 for the identification of the isolates).

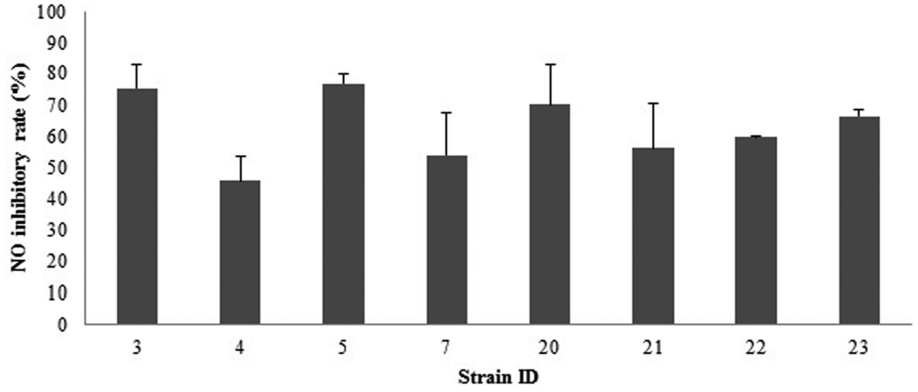

Fig. 2. Comparison of inhibitory rate (%) in NO production in the RAW 264.7 cells treated with the heat killed whole cells of 8 selected LAB strains (Strain ID: 3. *L. plantarum* CAU1054; 4. *L. plantarum* CAU1055; 5. *L. plantarum* CAU1064; 7. *L. plantarum* CAU1106; 20. *L. sakei* subsp. *sakei* CAU1245; 21. *L. sakei* subsp. *sakei* CAU1273; 22. *L. salivarius* CAU1301; 23. *L. salivarius* CAU1302).

modulating activities. Karthikeyan *et al*. (2013) reported that a solvent fraction extracted from a strain of *L. casei* (LC-EAS) can inhibit NO production by suppressing iNOS mRNA expression.

To obtain more insight into the anti-inflammatory effects exerted by selected *L. plantarum* strains, future studies are be needed to identify the active components in the bacterial cell structure and to verify the anti-inflammatory activity in animal models of disease and clinical experiments in humans.

Acid tolerance

Survival of probiotics during gastric transit is important for colonization of the GI tract. Therefore, resistance to low pH was examined as a first step in determining the probiotic potential of the LAB strains (McDonald *et al*., 1990). The survival rate of the LAB strains was investigated after 3 h of incubation in MRS broth at pH 3.0. As shown in Fig. 3, 17 strains showed survival rates above 90% after 3 h of incubation at low pH. Among these acid tolerant strains, 3 strains of *L. plantarum* (CAU1054, CAU1055, and CAU1064) and *L. salivarius* CAU1301 showed higher survival than the other strains (Fig. 3). In contrast, the viable counts of the *S. infantarius* subsp. *coli*, *W. cibaria*, *L. curvatus*, and *L. sakei* subsp. *sakei* strains were less than 1 Log CFU/mL after 3 h of exposure to pH 3.0.

Several *in vitro* assays have been developed for the selection of acid-resistant LAB strains, including incubation in gastric contents, exposure to pH-adjusted PBS, and the use of a dynamic model of the stomach (Alander *et al*., 1999). The acid tolerance test (conducted at pH 3.0) used in this study has been shown to be sufficient for screening acid-tolerant strains from many LAB isolates. Further-

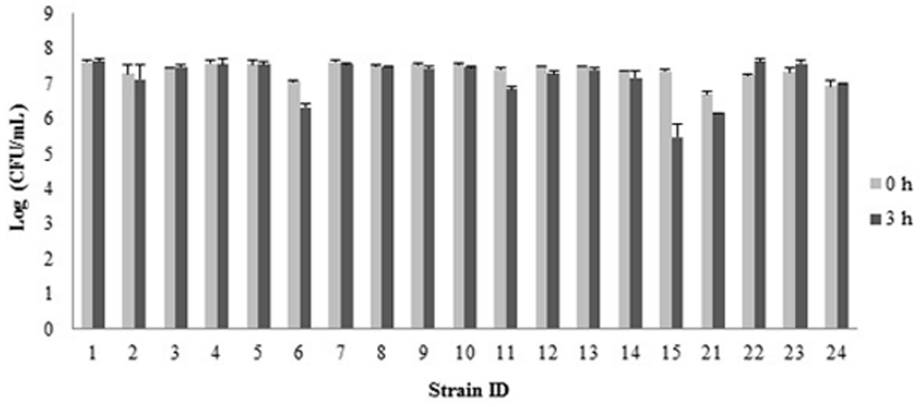

Fig. 3. Survival rates of lactic acid bacteria after 3 h exposure to pH 3.0 in MRS broth (Strain ID: See the Table 1 for the identification of the isolates). Data are an average of the data obtained by three independent experiments.

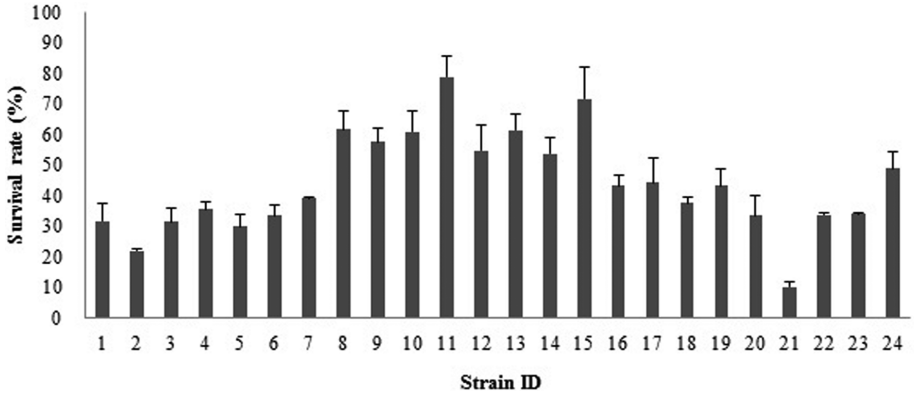

Fig. 4. Survival rates of the lactic acid bacteria after 12 h in MRS broth with 0.3% oxgall (Strain ID: See the Table 1 for the identification of the isolates).

more, Argyri *et al*. (2013) postulated that a pH value of 2.5 for the selection of potential probiotic strains is very selective because it is not the most common pH in the human stomach due to the buffering capacity of food or other carrier matrix molecules following consumption.

Bile tolerance

Bile salt tolerance is considered one of the properties required for the survival of probiotic strains in the small intestine. Bile secreted into the small intestine plays an important role in lipid digestion. However, due its amphiphilic nature, it is somewhat toxic to the GI microbiota because it can damage the lipid bilayer of bacterial cell membranes. The concentration of bile used here, 0.3%, is considered to be the critical concentration for screening resistant strains (Hyronimus *et al.*, 2000). As shown in Fig. 4, *P. pentosaceus*, *L. acidophilus*, *W. cibaria*, *W. viridescens* strains showed bile tolerance, whereas 1 strain of *L. sakei* subsp. *sakei* was the most susceptible to 0.3% oxgall. The bile tolerance of the selected LAB strains appears to be a strain-specific characteristic, as was reported previously (Mishra and Prasad, 2005).

Hydrophobicity

Bacterial cell surface hydrophobicity is important for the interactions between the bacterium and host intestinal epithelial cells that initiate bacterial adhesion in the GI tract. Previously, Perez *et al.* (1998) reported a high correlation between the hydrophobicity of the bacterial cell surface and adherence to intestinal epithelial cells. In the *in vitro* hydrophobicity test (Fig. 5), 7 strains showed greater than 50% hydrophobicity; 6 of them were members of the genus *Lactobacillus* and the remaining strain, 224, was identified as *W. viridescens*. These results are in a good agreement with those of Valeriano *et al.* (2010), who reported that all tested *Lactobacillus* strains showed

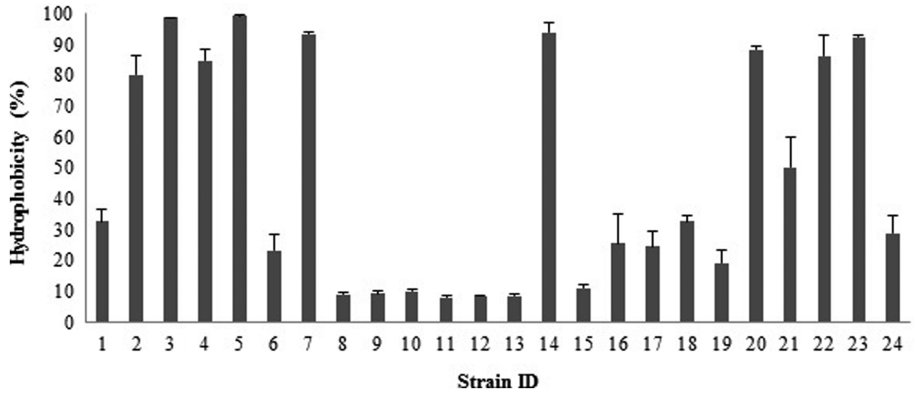

Fig. 5. Comparison of *in vitro* hydrophobicity of LAB isolates (Strain ID: See the Table 1 for the identification of the isolates).

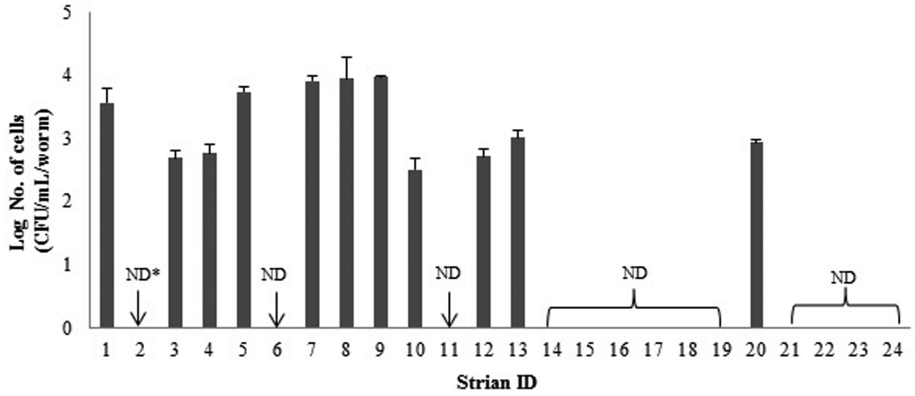

Fig. 6. Colonization of lactic acid bacteria on the *C. elegans* intestine (*Not detected) (Strain ID: See the Table 1 for the identification of the isolates).

hydrophobic surface characteristics (>50%), whereas some gram-negative bacteria, such as *E. coli* and *Salmonella* Typhimurium, exhibited relatively hydrophilic surfaces. Valeriano *et al.* (2014) also reported a significant correlation between *in vitro* adhesion on mucin, bacterial cell surface hydrophobicity, and auto-aggregation for the *L. mucosae* LM1 strain. However, these *in vitro* data may not correlate with the *in vivo* conditions due to various strain-specific mechanisms. Although some alternative *in vitro* adhesion assay models of the epithelial surface have been described, they have a critical limitation with regard to reflecting the actual human intestine.

C. elegans intestine adhesion

C. elegans is an accepted *in vivo* model to study bacteria-host interactions in the gut because the intestinal cells of this nematode worm are similar to those of humans (Park *et al*., 2014). Therefore, we used *C. elegans* as a surrogate *in vivo* screening system for potential probiotic LAB with the ability to adhere to the intestinal tract. As shown in Fig. 6, 5 strains (CAU1045, CAU1064, CAU 1106, CAU1212, and CAU1214) showed relatively high GI tract colonization. These strains exhibited outstanding persistence in the *C. elegans* intestine (>3.5 Log CFU/mL per worm). Another 6 strains (CAU1054, CAU1055, CAU1216, CAU1222, CAU1223, and CAU1245) also showed good colonization ability (2.5-3.0 Log CFU/mL per worm). However, the remaining strains were not able to colonize the intestinal tract of *C. elegans*. The results of the present study showed that *in vivo* colonization ability was a strain-specific characteristic. For example, 5 *L. plantarum* strains exhibited high colonization, whereas 1 *L. plantarum* strain (CAU1364) did not shown high colonization. Park *et al*. (2014) also reported that 4 strains of *L. plantarum* exhibited good colonizing ability, and these strains significantly extended the life span of *C. elegans* and enhanced their resistance to pathogenic bacteria such as *S. aureus*. It would be interesting to investigate the cor-

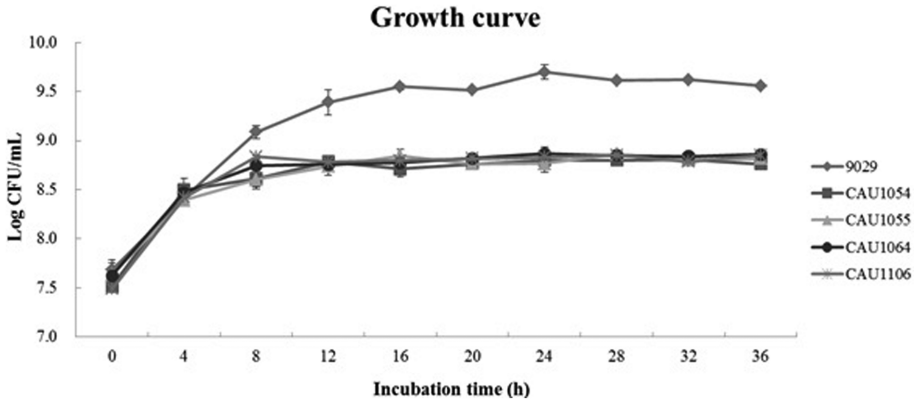

Fig. 7. Viable cell count of the *Lactobacillus plantarum* strains during the growth in 10% skim milk supplemented 2% glucose.

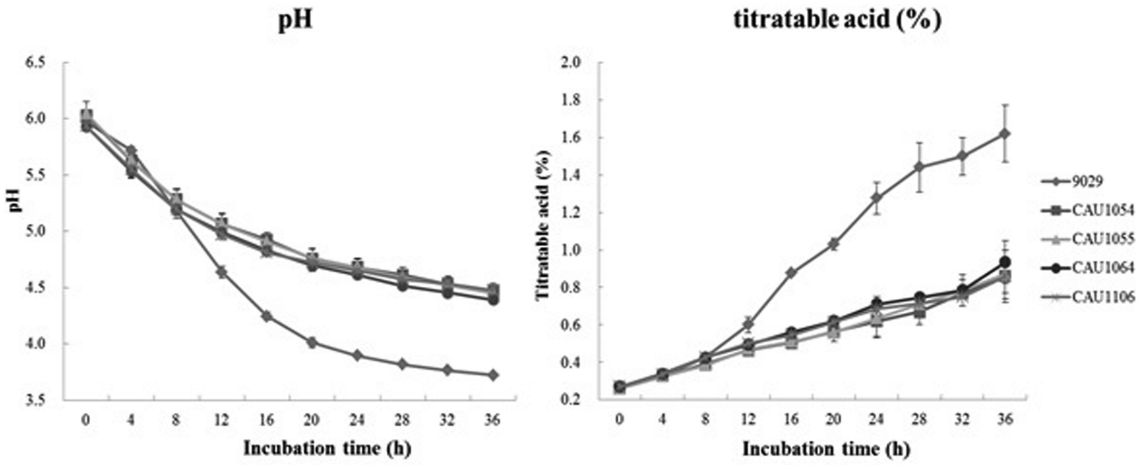

Fig. 8. The change of pH and titratable acidity of 10% skim milk supplemented with 2% glucose.

relation between colonizing ability and anti-inflammatory activity using good-colonizing and poor-colonizing *L. plantarum* strains in a future study.

Skim milk culture characteristics

Yogurt or fermented milk is one of the most efficient probiotic carrier foods. The milk fermenting ability of LAB strains is very important for use as a starter culture in dairy production. We investigated the growth characteristics of the *L. plantarum* strains that showed excellent anti-inflammatory activity (CAU1054, CAU1055, CAU1064, and CAU1106) in skim milk media. The selected strains were able to grow in skim milk media, reaching viable cell counts of 8.5 Log CFU/mL (Fig. 7), which was lower than that of *L. casei* YIT 9029 (also known as strain Shirota). Accordingly, the pH and TA values of the selected skim milk cultures were lower than those of *L. casei* YIT 9029 (Fig. 8). In order to use these probiotic candidates for yogurt production, it would be necessary to test for potential growth promoting conditions in milk and/or to co-culture them with other starter bacteria. Alternatively, these strains could be used in other probiotic applications with efficient delivery systems, including as lyophilized powders, capsules, and tablets.

Conclusions

We investigated the ability of selected probiotic candidate strains reduce NO production levels in cultures of LPS-stimulated RAW 264.7 macrophages. Based on morphological, biochemical, and molecular biological characterizations, the selected strains were identified as *L. plantarum*. The 4 *L. plantarum* strains isolated and characterized in this study meet the probiotic selection criteria for *in vitro* probiotic property assessment. Furthermore, they showed good colonization in a *C. elegans* intestine

in vivo model. Taken together, the selected *L. plantarum* strains show promise for use as probiotic agents and in fermented dairy food applications. Further studies are needed to obtain more insight into the anti-inflammatory effects of these strains in animal models and human clinical experiments.

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References

1. Alander, M., De Smet I., Nollet, L., Verstraete, W., von Wright, A., and Mattila-Sandholm, T. (1999) The effect of probiotic strains on the microbiota of the simulator of the human intestinal microbial ecosystem (SHIME). *Int. J. Food. Microbiol.* **46**, 71-79.
2. Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A. G., Tsakalidou, E., Nychas, G. J. E., Panagou, E. Z., and Tassou, C. C. (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* **33**, 282-291.
3. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.
4. Byrd, J. C. and Bresalier, R. S. (2004) Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev.* **23**, 77-99.
5. Carasi, P., Ambrosis, N. M., De Antoni, G. L., Bressollier, P., Urdaci, M. C., and Serradell Mde, L. (2014) Adhesion properties of potentially probiotic *Lactobacillus kefiri* to gastrointestinal mucus. *J. Dairy Res.* **81**, 16-23.
6. Choi, J. K., Lim, Y. S., Kim, H. J., Hong, Y. H., Ryu, B. Y., and Kim, G. B. (2012) Screening and characterization of *Lactobacillus casei* MCL strain exhibiting immunomodulation activity. *Korean J. Food Sci. An.* **32**, 635-643.
7. Chon, H., Choi, B., Lee, E., and Jeong, G. (2009) Immunomodulatory effects of specific bacterial component of *Lactobacillus plantarum* KFCC11389P on the murine macrophage cell line RAW 264.7. *J. Appl. Microbiol.* **107**, 1588-1597.
8. Duary, R. K., Rajput, Y. S., Batish, V. K., and Grover, S. (2011) Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.* **134**, 664-671.
9. Feighery, L. M., Smith, P., O'Mahony, L., Fallon, P. G., and Brayden, D. J. (2008) Effects of *Lactobacillus salivarius* 433 118 on intestinal inflammation, immunity status and *in vitro* colon function in two mouse models of inflammatory bowel disease. *Dig. Dis. Sci.* **53**, 2495-1506.
10. Food and Agriculture Organization of the United Nations. (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. (http://www.who.int/foodsafety/publications/fs_management/en/probiotics/pdf)
11. Gilliland, S. E. and Walker, D. K. (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in human. *J. Dairy Sci.***73**, 905-911.
12. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, J., Wishnok, J. S., and Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem*. **126**, 131-138.
13. Hedin, C., Whelani, K., and Lindsay, J. O. (2000) Evidence for the use of probiotics and probiotics in inflammatory bowel diseases: a review of clinical trials. *Proc. Nutr. Sor*. **66**, 307-315.
14. Hyronimus, B., Le Marrec, C., HadjSassi, A., and Deschamps, A. (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food Microbiol.* **61**, 193-197.
15. Jeong, I. Y., Lee, H. J., Jin, C. H., Park, Y. D., Choi, D. S., and Kang, M. A. (2010) Anti-inflammatory activity of *Stevia rebaudiana* in LPS-induced RAW 264.7 cells. *J. Food Sci. Nutr.* **15**, 14-18.
16. Karthikeyan, T., Pravin, M., Muthusamy, V. S., Raja, R. B., and Lakshmi, B. S. (2013) *In vitro* investigation of the immunomodulatory potential of probiotic *Lactobacillus casei*. *Probiotics Antimicro. Prot.* **5**, 51-58.
17. Kim, D. W., Cho, S. B., Yun, C. H., Jeong, H. Y., Chung, W. T., Choi, C. W., Lee, H. J., Nam I. S., Suh, H. H., Lee, S. S., and Lee, B. S. (2007) Induction of cytokines and nitric oxide in murine macrophages stimulated with enzymatically digested *Lactobacillus* strains. *J. Microbiol.* **45**, 373-378.
18. Kim, Y. and Mylonakis, E. (2012) *Caenorhabditis elegans* immune conditioning with the probiotic bacterium *Lactobacillus acidophilus* strain NCFM enhances gram-positive immune responses. *Infect. Immun.* **80**, 2500-2508.
19. Korhonen, R., Korpela, R., and Moilanen, E. (2002) Signalling mechanisms involved in the induction of inducible nitric oxide synthase by *Lactobacillus rhamnosus* GG, endotoxin, and lipoteichoic acid. *Inflammation* **26**, 207-214.
20. Lee, E. K., Lee, N. K., Lee, S. K., Chang, H. I., and Paik, H. D. (2010) Screening of immunostimulatory probiotic lactic acid bacteria from chicken feces as animal probiotics. *Korean J. Food Sci. An.* **30**, 634-640.
21. Lee, H. S., Han, S. Y., Bae, E. A., Huh, C. S., Ahn, Y. T., Lee, J. H., and Kim, D. H. (2008) Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice. *Int. Immunopharmacol.* **8**, 574-580.
22. Lee, S. K., Yang, K. M., Cheon, J. H., Kim, T. I., and Kim, W. H. (2012) Anti-inflammatory mechanism of *Lactobacillus rhamnosus* GG in lipopolysaccharide-stimulated HT-29 cell. *Korean J. Gastroenterol*. **60**, 86-93.
23. Leung, M. C. K., Williams, P. L., Benedetto, A., Au, C., Helmcke, K. J., Aschner, M., and Meyer, J. N. (2008) *Caenorhabditis elegans*: An emerging model in biomedical and environmental toxicology. *Toxicol. Sci.* **106**, 5-28.

24. Lorsbach, R. B., Murphy, W. J., Lowenstein, C. J., Snyder, S. H., and Russell, S. W. (1993) Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing: molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* **268**, 1908-1913.
25. Maldonado, G. C., Doreno, D., de LeBlanc, A., Vinderola, G., Bibas, B., Perdigo'nG, M. E. (2007) Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria. *Clin. Vaccine Immunol.* **14**, 485-492.
26. Mcdonald, L. C., Fleming, H. P., and Hassan, H. M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum. Appl. Environ. Microbiol.* **56**, 2120-2124.
27. McGhee, J. D. (2007) The *C. elegans* intestine. WormBook **27**, 1-36.
28. Mishra, V. and Prasad, D. N. (2005) Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int. J. Food Microbiol.* **103**, 109-115.
29. Ouwehand, A. C. and Salminen, S. (2003) *In vitro* adhesion assays for probiotics and their *in vivo* relevance: a review. *Microb. Ecol. Health Dis.* **15**, 175-184.
30. Park, M. R., Yun, H. S., Son, S. J., Oh, S., and Kim, Y. (2014) Development of a direct *in vivo* screening model to identify potential probiotic bacteria using *Caenorhabditis elegans. J. Dairy Sci.* **97**, 6828-6834.
31. Perez, P. F., Minnaard, Y., Disalvo, E. A., and Antoni, G. L. (1998) Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microb.* **64**, 21-26.
32. Valeriano, V. D., Parungao-Balolong, M. M., and Kang, D. K. (2014) *In vitro* evaluation of the mucin-adhesion ability and probiotic potential of *Lactobacillus mucosae* LM1. *J. Appl. Microbiol.* **117**, 485-497.
33. Van Tassell, M. L. and Miller, M. J. (2011) *Lactobacillus* adhesion to mucus. *Nutrients* **3**, 613-636.

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