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The Potential Probiotic and Functional Health Effects of Lactic Acid Bacteria Isolated from Traditional Korean Fermented Foods

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This study investigated the probiotic properties and physiological activities of Korean fermented foods such as sikhae, young radish kimchi, and bean-curd dregs. Among the isolated lactic acid bacteria, *Pediococcus inopinatus* BZ4, *Lactobacillus plantarum* SH1, *Lactobacillus brevis* SH14, *Pediococcus pentosaceus* YMT1, and *Leuconostoc mesenteroides* YMT6 demonstrated a greater than 60% survival rate at pH 2.5, along with an excellent survival rate even at 0.3% bile acid. These five bacteria showed strong flocculation ability in autoaggregation and coaggregation tests, indirectly clustering useful micro-organisms and inhibiting the attachment of pathogenic bacteria. In a cell surface hydrophobicity test, these bacteria showed adhesion to three solvents (ethyl acetate, chloroform, and xylene) and high hydrophobicity, thereby indicating excellent indirect cell adhesion to intestinal cells. The cell-free supernatants and intracellular extracts of the five lactic acid bacteria showed antioxidative activity in the form of 2,2-Diphenyl-1-picrylhydrazyl and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging ability and lipid peroxidation inhibition. Antimicrobial activities were also observed in four pathogenic bacteria, namely *E. coli* KCTC 2571, *H. pylori* HPKCTC B0150, *L. monocytogenes* KCTC 13064, and *S. aureus* KCTC 1916. These results demonstrate that these five lactic acid bacteria could be used as probiotics with antioxidant and antimicrobial properties.

Key words : Antimicrobial activity, antioxidative activity, lactic acid bacteria, probiotics

Introduction

In some countries, including the Republic of Korea, the average life expectancy had seen a rapid growth due to advancements in medical conditions, living standards, and the environment, further leading to an aging society. Therefore, various foods and medicines have been studied to maintain a good health until old age. However, synthetic foods can cause side effects due to toxicity, even if they are effective. Recently, research on natural materials along with well-being culture has garnered attention as the mainstream of the future food industry along with an increased interest in lactic acid bacteria [14]. Lactic acid bacteria are the main microorganisms in fermented food, which uses sugar to metabolize them to produce organic acids and bacteriocins. The use of lactic acid bacteria in lactose intolerance, intestinal regulation, immunomodulation, anticancer, antimutagenic effect, cholesterol-lowering, and antiallergenic effects has increased rapidly in the industry [23]. In general, lactic acid bacteria have a tolerance to lower pH in the intestines and bile acids while having stability as that of GRAS. Therefore, the mainstream use of lactic acid bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, for probiotics has been reported [26]. To use microorganisms as probiotics, it is necessary to identify their various functions, safety issues, and technical factors [13]. It must survive at low pH and the presence of bile acids, reach the intestine, settle, and colonize. In other words, stability, which is the viability of the strain, must be considered first and these bacteria are known to have the ability to adhere to the epithelial cells and aggregate further [18]. Also, these bacteria should be harmless to the human body and have no side effects. Oxidative stress causes biochemical reactions and diseases that are harmful to living organisms. During the respiratory metabolism, a certain amount of oxygen generates harmful substances known as reactive oxygen species (ROS). These ROS are known to destroy biological tissues and cells via strong oxi-

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dative forces. Lactic acid bacteria have an antioxidant mechanism that protects them from free radicals, and studies have reported the antioxidant effects of lactic acid bacteria [16]. The harmful bacteria, including *Candida sp.*, *Escherichia coli*, *Helicobacter pylori*, *Listeria monocytogenes*, and *Staphylococcus aureus*, that can cause food poisoning and inflammation, have been reported to be the most common cause of candidemia, especially *Candida albicans* [9]. *E. coli* is a pathogenic bacterium that causes diarrhea and death in infants and young children [5]. *H. pylori* is known to cause chronic gastritis, gastric ulcer, mucosa-associated lymphoid tissue tumor, and gastric cancer [11]. *S. aureus* is one of the bacteria widely present in nature and produces enterotoxins that causes food poisoning. This bacterium cause skin suppuration, ear infections, and cystitis [25]. *L. monocytogenes* induces miscarriage and stillbirth in pregnant women and listeriosis in neonates, infants, and adults with an impaired immune function, such as pneumonia, endocarditis, sepsis, abscess, and conjunctivitis [12]. Lactic acid bacteria inhibit the intestinal adhesion and colonization of these harmful bacteria or produce antimicrobial substances such as bacteriocin to reduce the production of decay products and prevent diseases. Therefore, in this study, we tested acidic and bile resistance, cell surface hydrophobicity, and aggregation reaction to isolate lactic acid bacteria from Korean traditional fermented foods and characterize them as probiotics. Also, we screened and identified strains with excellent antioxidant and antimicrobial activities to verify their applicability as a functional probiotic material.

Materials and Methods

Isolation of lactic acid bacteria

Sikhae and young radish kimchi were obtained from a house in Pohang, Korea. The bean-curd dregs were obtained from a house in Hongseong, Korea. In total, 10 g of each sample was mixed with 10 ml of sterilized distilled water and centrifuged at 3,100× g for 10 min (1248R, GYROZEN Co., Ltd., Korea). The supernatant is diluted stepwise and inoculated onto the agar plates of *Lactobacilli* de Man, Rogosa, and Sharpe (MRS, Difco, USA) and cultured at 30℃ for 48 hr. Single colonies of each sample were inoculated onto the Bromocresol purple (BCP, Difco, USA) agar and cultured at 30℃ for 24 hr to select lactic acid bacteria that form a yellow circle. The selected colonies were subcultured in MRS broth for activation and stored at -70℃ in 20℃ glycerol stock.

Evaluation of tolerance to acid and bile salt

Acid tolerance was studied according to the method of Tokatlı *et al* [24]. Lactic acid bacteria cultured in the MRS broth for 24 hr at 30℃ were centrifuged at 6,000× g for 10 min with centrifugation for cell separation. The pellet was washed twice with phosphate-buffered saline (PBS) and re-suspended in PBS adjusted to the pH of 2.5. The suspension was incubated at 30℃ for 4 hr and the suspension at 0 and 4 hr of culture was diluted with sterilized saline. The diluted culture is plated on the MRS agar at 30℃ for 24 hr. The survival rate of lactic acid bacteria was calculated according to equation (a). Bile salt tolerance was studied according to the method of Tokatlı *et al* [24]. Lactic acid bacteria cultured in MRS broth for 24 hr at 30℃ were centrifuged at 6,000× g for 10 min for cell separation. The pellet was washed twice with PBS and incubated 1% MRS broth with 0.3% (w/v) oxgall (Difco, USA) for 4 hr at 30℃. The suspension at 0 and 4 hr of culture was diluted with sterilized saline and plated on the MRS agar at 30℃ for 24 hr. The survival rate of the lactic acid bacteria was calculated according to the following equation:

$$\text{(a) Survival (\%)} = \frac{\text{logCFU of viable cells survived}}{\text{logCFU of initial viable cells inoculated}} \times 100$$

Autoaggregation and coaggregation assay

Autoaggregation and coaggregation abilities of isolated lactic acid bacteria were measured by Raouf *et al* method [22]. The pathogenic strain *C. albicans* ATCC 10231 was obtained from the American Type Culture Collection (ATCC, USA). *E. coli* KCTC 2571, *L. monocytogenes* KCTC 13064, and *S. aureus* KCTC 1916 were obtained from the Korean Agricultural Culture Collection (Korea). *H. pylori* HPKCTC B0150 was obtained from the *H. pylori* Korean Type Culture Collection (HPKTCC, Korea). Lactic acid bacteria and pathogenic strain were washed twice with PBS and the OD_{600} value was adjusted to 0.3 by using the same buffer. The OD_{600} was measured at 0, 1, 2, 3, 4, 5, 10, 20, and 24 hr at room temperature using a spectrophotometer (Libra S22, Biochrom Ltd., England). Autoaggregation was calculated according to the following equation:

$$\text{Autoaggregation (\%)} = \frac{1 - OD_{time}}{OD_{T0}}$$

To measure the coaggregation ability of lactic acid bacteria, the lactic acid bacteria and pathogenic strain were treated in the same manner as the autoaggregation test. The OD_{600} of a mixture of lactic acid bacteria and pathogenic

strain at the same rate was measured at 0, 1, 2, 3, 4, 5, 10, 20, and 24 hr at room temperature. Coaggregation was calculated according to the following equation:

$$\text{Coaggregation (\%)} = \frac{(OD_{Patho} + OD_{Lb})/2 - OD_{mix}}{(OD_{Patho} + OD_{Lb})/2} \times 100$$

Evaluation of Cell surface hydrophobicity

To indirectly confirm the adhesion ability of lactic acid bacteria in the intestines of human beings, the cell surface hydrophobicity was studied according to the research of Doyle *et al* [10]. The ability of hydrophobicity was measured based on the adhesion of cells to organic solvents. Lactic acid bacteria were cultured at 30℃ for 18 hr, and then centrifuged at 3,100× g for 10 min to separate the cells. The pellet was washed twice with PBS and the OD_{600} measurement value was adjusted to 1.0. The equal volume of chloroform, ethyl acetate, and xylene were mixed by vortexing. Chloroform was used as a monopolar and acidic solvent, ethyl acetate was used as a monopolar and basic solvent, and xylene was used as a non-polar solvent. The mixture was allowed to stand for 30 min at room temperature. When the layers were separated, the absorbance of the suspension and the organic solvent was measured at a wavelength of 600 nm. The hydrophobicity (%) was calculated according to the following equation:

$$\text{Hydrophobicity (\%)} = \frac{\text{Absorbance of sample - Absorbance of solvent}}{\text{Absorbance of solvent}} \times 100$$

Free radical scavenging activity

The lactic acid bacteria used for the measurement of antioxidant activity were prepared according to the method of Chen *et al.* [4]. The lactic acid bacteria were incubated in MRS broth at 30℃ overnight, and each of lactic acid bacteria cell pellets was separated by centrifuge at 3,100× g for 10 min. The cell-free supernatant was obtained by filtration with a syringe filter (0.45 μM, E.Chrom Science, Korea), and the pellet was washed twice with PBS and re-suspended in the same buffer. The bacterial counts in the cell suspension were adjusted to 10^8 CFU/ml. Thereafter, the cell suspensions were disrupted with an ultrasonic wave (S-450D, Branson Ultrasonics Co., USA) for 10 min on an ice bath, followed by centrifuge at 3,100× g for 10 min, and the supernatant, which was the intracellular cell-free extract, was collected. The free radical scavenging activity of 2,2-Diphenyl-1-picryhydrazyl (DPPH) was measured by the method of Brand-Williams *et al* [1]. In total, 800 μl of cell-free supernatants and intracellular cell-free extracts (LAB samples) were mixed 1 ml of DPPH solution (200 μM in methanol), and the mixture was reacted at room temperature for 30 min. After the reaction, the absorbance was measured with a spectrophotometer at 517 nm. For positive control, butylated hydroxytoluene (BHT, Sigma Co., USA) was used. The radical scavenging activity (%) of DPPH was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Next, we measured the radical scavenging activity of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) according to the work of Pellegrin *et al* [20]. In all, 5 ml of 7 mM ABTS solution and 88 μl of $K_2S_2O_8$ solution are mixed and allowed to stand in a dark room for 14-16 hr to make a stock solution. Then, 1 ml of ABTS solution prepared by mixing 1 ml of stock solution and 88 ml of ethanol with 50 μl of LAB samples. After vortexing, the solution was reacted for 2 min and 30 seconds, and absorbance was measured at a wavelength of 734 nm. The radical scavenging activity (%) of ABTS was calculated according to the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Lipid peroxidation inhibition activity

The activity of lipid peroxidation was determined by thiobarbituric acid (TBA) method, which is based on the peroxidation of linoleic acid and according to Kong *et al* [17]. Linoleic acid emulsion was prepared by mixing 0.1 ml of linoleic acid and 0.2 ml of Tween 20 in distilled water. In total, 1 ml of the emulsion solution, 0.4 ml of the LAB samples, 0.5 ml of sodium phosphate buffer (0.02 M, pH 7.4), and 0.2 ml of 1% $FeSO_4$ were mixed and reacted at 37℃ for 1 hr. To this combined solution, we added 0.2 ml of 4% trichloroacetic acid, 0.8% TBA, and 0.4% BHT. The mixture was reacted for 30 min, cooled, and then 2 ml of butanol was added. After vortexing for 1 min, the mixture is centrifuged at 1,800× g for 15 min. Thereafter, the supernatant was centrifuged under the same conditions. After standing at room temperature, the absorbance of the supernatant was measured at a wavelength of 532 nm. The inhibition rate of lipid peroxidation (%) was calculated according to the following equation:

$$\text{Inhibition rate of lipid peroxidation (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Antimicrobial and antifungal activity

The antimicrobial and antifungal test was performed according to the disc diffusion assay described by Chang *et al* [2]. The activity of lactic acid bacteria was tested against the following microorganisms: *C. albicans* ATCC 10231, *E. coli* KCTC 2571, *H. pylori* HPKCTC B0150, *L. monocytogenes* KCTC 13064, and *S. aureus* KCTC 1916. Each pathogenic strain was cultivated under optimal conditions. The pathogen culture was washed twice with PBS and the OD_{600} value was adjusted to 0.1 by using the same buffer. The pathogens were inoculated at 1% on Mueller Hinton (Difco, USA) agar. The cell-free supernatant of the lactic acid bacteria was dispensed in a volume of 60 μl onto the sterilized paper disc (8 mm, Toyo Roshi Kaisha, Ltd., Japan) and attached on the agar plate of the pathogenic strain. After incubation at 30℃ for 48 hr, the diameters of the inhibitory zones were measured in millimeters. Ampicillin and amphotericin B were used as positive controls.

Morphological characterization

Before molecular biological identification, gram staining was performed to increase the efficiency of primer. Selected lactic acid bacteria were stained with a gram staining kit (Difco, USA). According to the cell wall structure, the shape and size of the strain, the staining results were observed under an optical microscope.

Molecular biological identification

16S rDNA sequencing was performed on first selected strains to confirm the change of lactic acid bacteria. Phenol-chloroform method [15] was used to isolate the chromosomal DNA of the strains. In all, 2 μl of 10X Taq buffer, 1.6 μl of 2.5 mM dNTPs, 1 μl of primers (forward and reverse each), template DNA 20 ng, and Taq polymerase 0.2 U (LPS solution, Korea) were mixed to prepare a polymerase chain reaction (PCR) reaction solution. After initial denaturation at 95℃ for 5 min, the processes of denaturation at 95℃ for 1 min, annealing at 55℃ for 30 seconds, and extension at 72℃ for 1 min were repeated 35 times, followed by final extension at 72℃ for 5 min. Amplification products were identified by 1.5% agarose gel electrophoresis. The primers used in the PCR reaction were prepared according to the methods of Petri *et al* [21].

Biochemical identification

The isolated lactic acid bacteria were subcultured on MRS broth, and the availability of 49 carbon sources was confirmed through API 50 CHL kit (Biomérieux, France). Api web program (http://apiweb.biomerieux.com) was used to read proximity to standard species and finally identified.

Statistical analysis

All data were analyzed statistically by using the Statistical Package for Social Sciences (SPSS, version 23.0, SPSS Inc., USA) and presented as mean ± standard deviations (SD). One-way analysis of variance with the Duncan's multiple range test used to measure the significant differences between values ($p<0.05$).

Results

Acid and bile salt tolerance

For lactic acid bacteria to have a beneficial effect on the host, it must pass through a strong acidic stomach and then through the duodenum, where the bile is present, to reach the target area. The pH of the stomach is generally 2.5-3.5, and most microorganisms are killed in stomach acid. Therefore, lactic acid bacteria should exhibit acid tolerance to stomach acid to survive at low pH environment maintained by gastric acid. Fig. 1 presents the abilities of the lactic acid bacteria to tolerate the artificial gastric juice. The survival rates of all strains declined over the 4 hr of incubation and these strains tolerated a pH of 2.5 for 4 hr. however, they did that with different survival rates. The survival rate of BZ4 was 68.37%, which is the best obtained value among the strains. SH1, SH14, YMT1, and YMT6 showed more than 60% survival rate and was significantly different from other strains. Some lactic acid bacteria have been reported to neutralize pH stress through their ability to regulate intracellular pH. The mechanism by which lactic acid bacteria can tolerate acidic conditions by the activation of proton pump by ATPase. Therefore, it is considered that BZ4, SH1, SH14, YMT1, and YMT2 show excellent tolerance to acid by their ability described above. On the contrary, tolerance to bile acids is one of the basic characteristics that probiotic microorganisms must possess in addition to tolerance to acidic conditions mentioned above. Bile is an amphipathic molecule and has a strong antimicrobial activity that destroys biological membrane. The bile salt at a concentration of 0.3% (w/v) is the average concentration of bile salts in the human intestines. Fig. 2 presents the results of incubation for 4 hr in the 0.3% (w/v) oxgall-supplemented medium.

Most of the strains showed a survival rate of over 40%. Among them, the strains (BZ4, SH1, SH14, YMT1, and YMT2) that showed strong tolerance to acid had a significantly higher survival rate even in bile salt. Based on these results, the following experiments were performed using five lactic acid bacteria (BZ4, SH1, SH14, YMT1 and YMT2) showing excellent results in the evaluation of tolerance to acid and bile salt.

Autoaggregation and coaggregation

Aggregation is the process in which bacteria physically interact with each other. Probiotics interact with other intestinal microbes in the human intestinal tract. Colonization and interaction of lactic acid bacteria inhibit pathogens by inhibiting the biofilm formation of pathogens and adherence to the intestinal membrane cells. The aggregation ability of lactic acid bacteria is correlated with adhesion to epithelial cells and evaluates the adherence ability indirectly [6]. As

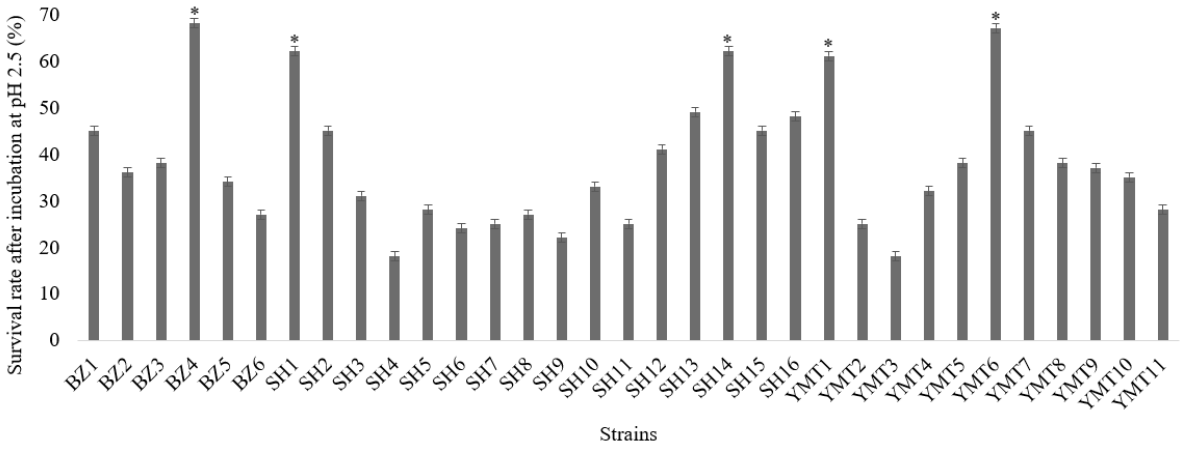

Fig. 1 Acid tolerance of lactic acid bacteria isolated from Korean traditional fermented foods. BZ, SH, and YMT were lactic acid bacteria isolated from bean-curd dregs, sikhae, and young radish kimchi, respectively. Lactic acid bacteria were suspended in PBS buffer (pH 2.5) and reacted for 4 hr at 30 ℃. The survival rate was calculated by counting the viable cell of 0 and 4 hr.

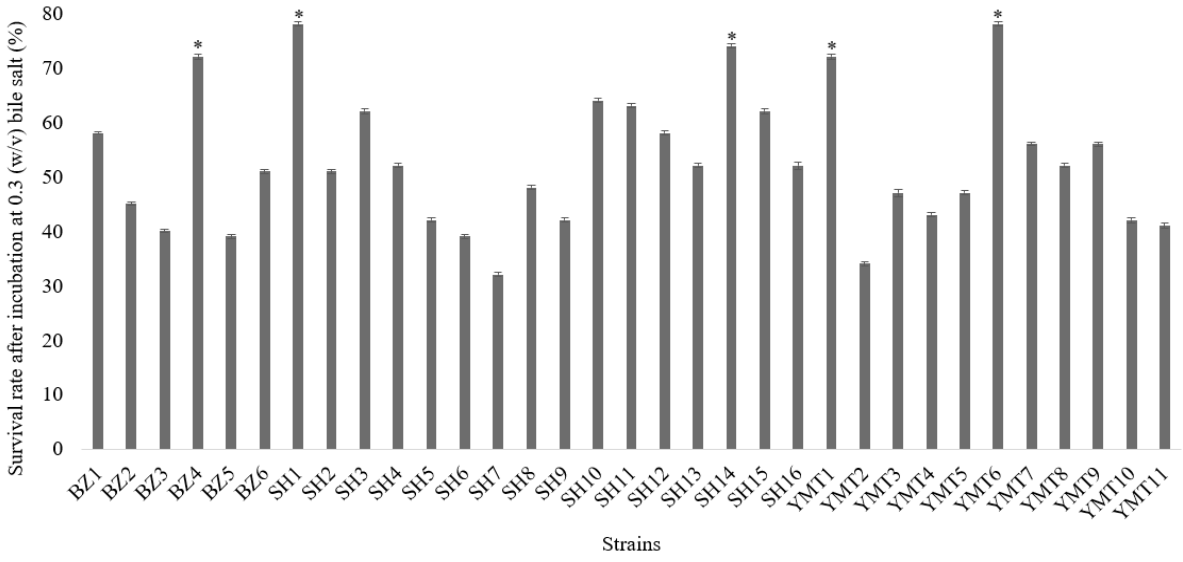

Fig. 2 Bile tolerance of lactic acid bacteria isolated from Korean traditional fermented foods. Lactic acid bacteria were incubated in MRS medium containing 0.3% bile acid at 30℃ for 4 hr. The survival rate was calculated by counting the viable cell of 0 and 4 hr. As a result of acid and bile tolerance experiments, five lactic acid bacteria (*P. inopinatus* BZ4, *L. plantarum* SH1, *L. brevis* SH14, *P. pentosaceus* YMT1, and *Lc. mesenteroides* YMT6) with the highest survival rates were selected and the following experiments were conducted.

shown in Table 1, these values increase based on the incubation time. At 1 hr, the values of all strains were similar, but there was a difference from 10 hr. In particular, YMT6 rapidly aggregated from 5 hr and exhibited the best autoaggregation ability among strains, except for *C. albicans* at 24 hr. The autoaggregation mechanism is not precisely defined but may be due to cell surface charge and composition. In addition, Table 2 shows the results of coaggregation of lactic acid bacteria with pathogens. The ability to coaggregation with *C. albicans* was highest in SH1, with the value of 67.94±0.68%, which was the highest among all strains. These values were followed by that of BZ4 and YMT6, which were 60.63±0.62% and 56.52±0.31%, respectively. In the coaggregation ability with *H. pylori*, all lactic acid bacteria, except YMT1, showed the excellent value of about 60%. *E. coli*, *L. monocytogenes*, and *S. aureus* showed coaggregation abilities from 43.70±0.82% to 52.63±0.93% in all lactic acid bacteria. All the tested lactic acid bacteria showed the ability to aggregate with pathogens after 24 hr of incubation. however, the percentage of coaggregation was strain specific and dependent on time and incubation conditions.

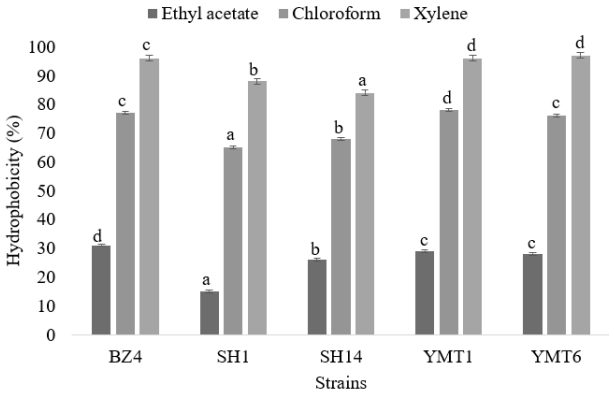

Fig. 3. Cell surface hydrophobicity of selected lactic acid bacteria. Lactic acid bacteria were suspended in PBS buffer and the OD value was set to 1.0. The suspension was mixed with the same amount of solvent (chloroform, ethyl acetate, and xylene) and left at room temperature for 30 min. When the layers are separated, hydrophobicity is calculated using the absorbance of organic solvent and suspension. Means with differed letters (a - d) above the bars in the same material are significantly different at $p<0.05$, as confirmed by Duncan's multiple range test. The data were expressed as mean ± SD.

Cell surface hydrophobicity

It has been reported that the adhesion properties of lactic acid bacteria to cells are related to the cell surface charge and hydrophobicity of bacteria and their ability to adhere to hydrocarbons can be expressed as cell surface hydrophobicity. Hydrophobicity can provide a competitive advantage for adherence to the intestinal epithelial cells as long distance non-covalent interactions. Fig. 3 shows the hydrophobicity of lactic acid bacteria. Lactic acid bacteria showed higher hydrophobicity in chloroform than ethyl acetate, thereby indicating strong electron donors. The adhesion value of all lactic acid bacteria to xylene was higher than 83%, especially YMT1 and YMT6 showed a significant difference with high hydrophobicity of 97% or more. Therefore, it is considered that the adhesion of the lactic acid bacteria is improved due to the excellent hydrophobicity.

Table 1. Autoaggregation of selected lactic acid bacteria and pathogenic strains

Strains	% of autoaggregation (25℃)			
	1 hr	5 hr	10 hr	24 hr
Pathogenic strains				
C. albicans ATCC 10231	1.28±0.17^{a}	10.39±0.34^{f}	43.42±4.97^{e}	87.43±0.55^{i}
E. coli KCTC 2571	1.42±0.37^{a}	5.82±0.31^{ab}	21.10±0.92^{c}	60.60±0.82^{g}
H. pylori HPKCTC B0150	1.45±0.36^{a}	6.21±0.23^{b}	17.07±0.15^{b}	49.61±0.80^{f}
L. monocytogenes KCTC 13064	1.47±0.28^{a}	5.26±0.35^{a}	15.28±0.53^{ab}	32.46±0.51^{b}
S. aureus KCTC 1916	1.32±0.21^{a}	7.20±0.26^{c}	14.41±0.25^{ab}	21.41±0.78^{a}
Lactic acid bacteria				
BZ4	1.51±0.59^{a}	7.97±0.79^{d}	13.19±0.34^{a}	38.30±0.57^{c}
SH1	2.27±0.32^{b}	10.72±0.14^{f}	20.74±0.17^{c}	44.43±0.55^{e}
SH14	1.47±0.44^{a}	8.69±0.33^{e}	17.45±0.33^{b}	41.76±0.98^{d}
YMT1	1.39±0.33^{a}	8.75±0.56^{e}	15.22±0.39^{ab}	37.33±0.41^{c}
YMT6	1.17±0.32^{a}	17.14±0.48^{g}	26.25±0.38^{d}	62.11±0.31^{h}

(a - i) Significant differences ($p<0.05$) among all bacteria strains were tested at same time. The data were expressed as the mean ± SD (n=3).

Table 2. Coaggregation of selected lactic acid bacteria and pathogenic strains

Strains	% of coaggregation (25℃)			
	1 hr	5 hr	10 hr	24 hr
Coaggregation with *C. albicans* ATCC 10231				
BZ4	6.79±0.47^{b}	18.20±0.05^{c}	38.09±0.65^{d}	60.63±0.62^{c}
SH1	4.58±1.17^{a}	18.17±0.32^{c}	44.62±0.40^{e}	67.94±0.68^{d}
SH14	5.84±0.57^{b}	11.45±0.50^{b}	31.03±0.52^{b}	53.26±0.35^{b}
YMT1	4.20±0.90^{a}	9.12±0.69^{a}	16.42±1.09^{a}	25.56±0.84^{a}
YMT6	6.75±0.33^{b}	12.00±0.67^{b}	34.23±0.51^{c}	56.52±0.31^{c}
Coaggregation with *E. coli* KCTC 2571				
BZ4	5.64±1.07^{b}	9.92±0.84^{c}	24.81±0.30^{b}	46.07±0.75^{b}
SH1	4.40±1.01^{ab}	11.29±0.17^{a}	27.61±0.41^{c}	48.91±0.66^{c}
SH14	4.37±1.14^{ab}	8.91±0.83^{bc}	25.54±0.58^{b}	46.98±0.83^{b}
YMT1	3.63±0.32^{a}	6.20±0.99^{a}	23.60±0.40^{a}	45.96±0.85^{b}
YMT6	5.66±0.81^{b}	8.00±0.20^{b}	23.84±0.66^{a}	44.08±0.57^{a}
Coaggregation with *H. pylori* HPKCTC B0150				
BZ4	6.50±0.45^{c}	13.61±0.39^{b}	32.06±0.22^{e}	58.69±0.45^{c}
SH1	6.47±0.38^{c}	13.30±0.29^{b}	31.08±0.45^{d}	61.90±0.21^{e}
SH14	5.50±0.43^{b}	14.44±0.41^{d}	30.21±0.52^{c}	60.11±0.33^{d}
YMT1	4.47±0.36^{a}	8.84±0.36^{a}	26.80±0.37^{b}	48.17±0.28^{b}
YMT6	5.50±0.43^{b}	14.44±0.41^{d}	30.21±0.52^{c}	60.11±0.33^{d}
Coaggregation with *L. monocytogenes* KCTC13064				
BZ4	4.45±0.16^{a}	9.15±0.39^{a}	25.67±1.02^{b}	46.55±0.23^{b}
SH1	5.52±0.63^{a}	14.62±0.39^{d}	30.82±0.74^{d}	52.08±0.22^{e}
SH14	5.01±0.85^{a}	10.69±0.48^{b}	27.24±0.49^{c}	48.22±0.35^{c}
YMT1	5.68±0.34^{a}	12.44±0.45^{c}	28.34±0.52^{c}	49.97±0.23^{d}
YMT6	5.04±1.07^{a}	8.31±0.62^{a}	23.94±0.29^{a}	45.50±0.59^{a}
Coaggregation with *S. aureus* KCTC1916				
BZ4	4.68±0.55^{a}	7.30±0.13^{a}	21.75±0.48^{a}	46.81±0.26^{b}
SH1	4.44±0.60^{a}	11.51±0.70^{c}	27.77±0.67^{d}	52.63±0.93^{c}
SH14	4.97±0.17^{a}	7.67±0.52^{a}	22.04±0.65^{a}	46.53±0.55^{b}
YMT1	4.87±0.25^{a}	9.27±0.46^{b}	25.15±0.49^{c}	47.56±1.03^{b}
YMT6	4.37±0.46^{a}	7.34±1.06^{a}	23.54±0.50^{c}	43.70±0.82^{a}

(a - i) Significant differences (p<0.05) among all bacteria strains were tested at the same time. The data were expressed as the mean ± SD (n=3).

Free radical scavenging activity

Oxidative stress leads to several chronic diseases in humans, which are ultimately caused by active oxygen. Oxidation is a reaction that transfers electrons from a material to an oxidant. Oxidation reactions can generate free radicals with non-covalent electrons that cause rapid chain reactions, further destabilizing other molecules and producing additional free radicals. At this time, the antioxidant converts electrons or hydrogen atoms to free radicals to form irreversible and stable molecules. The antioxidant activity can be measured through electron-donating ability. Two types of radical scavenging activity assays were used to improve the validity of the results. DPPH has a free radical and when it reacts with a substance having an antioxidative activity, the radical is extinguished by giving out electrons, and the specific purple color becomes transparent and yellowish. In addition, higher radical scavenging activity indicates a higher antioxidant activity. The DPPH radical scavenging activity of cell-free supernatants and intracellular cell-free extracts of lactic acid bacteria (Table 3) and the supernatant of BZ4, SH1, and SH14 (highest activity) were 82.71±0.70%, 83.41±1.04%, and 82.50±1.38%, respectively, which was approximately 8% higher than antioxidant activity (74.59±0.86%) at 100 μg/ml of synthetic antioxidant BHT. The antioxidant activity of YMT1 was 73.55±1.77%, which was not significantly different from that of BHT 100 μg/ml. Intracellular cell-free extracts were also found to have a DPPH radical scavenging ability of 23% or more; however, they were relatively low as compared to the supernatant. Next, 2,2'- ABTS radical is cleared by antioxidant power, and the unique cyan color is

Table 3. DPPH radical scavenging activity of selected lactic acid bacteria

Strain	DPPH radical scavenging activity (%)	
	Cell-free supernatants	Intracellular cell-free extracts
BZ4	82.91±0.70^{C}	36.77±0.70^{d}
SH1	83.41±1.04^{C}	31.92±0.30^{c}
SH14	82.50±1.38^{C}	27.12±0.86^{b}
YMT1	73.55±1.77^{B}	27.71±0.50^{b}
YMT6	68.73±0.63^{A}	23.86±0.52^{a}
BHT*	74.59±0.86^{B}	74.59±0.86^{e}

(A – C) Means within the same sample (cell-free supernatants). (a – e) Means within the same sample (intracellular cell-free extracts) above the bars are significantly different at $p<0.05$, as confirmed by Duncan's multiple range test. The data were expressed as mean ± SD.
*BHT concentration: 100 μg/ml

discolored. This decolorization reaction is a method of measuring the degree of elimination of free radicals by absorbance and is completed within 1 min and can be measured in a short time. In this experiment, ABTS radical scavenging activity of lactic acid bacteria was measured (Table 4). In the cell-free supernatants, the ABTS radical scavenging activity of BZ4 was 62.98±1.14, which was lower than that of BHT 100 μg/ml (73.87±0.26%); however, it showed the highest activity among the lactic acid bacteria. Cell-free extracts also exhibited ABTS scavenging activity, but the figure was below 20%. This result shows that the cell-free supernatant was significantly higher than that of intracellular cell-free extracts as well as DPPH radical scavenging activity. Lactic acid bacteria have been reported to have an antioxidant activity that can protect themselves from active oxygen. The antioxidative effect of lactic acid bacteria is also related to the production of tocopherol, glutathione, and other removers. Also, teichoic acid and peptidoglycan of lactic acid bacteria membrane also affect the active oxygen scavenging. Therefore, it is considered that the aforementioned lactic acid bacteria can prevent various diseases induced by active oxygen.

Table 4. ABTS radical scavenging activity of selected lactic acid bacteria

Strain	ABTS radical scavenging activity (%)	
	Cell-free supernatants	Intracellular cell-free extracts
BZ4	62.98±1.14^{C}	13.32±0.61^{b}
SH1	60.61±0.60^{B}	17.30±1.59^{c}
SH14	57.05±0.60^{A}	12.86±0.51^{b}
YMT1	57.05±0.60^{A}	11.18±0.93^{a}
YMT6	56.92±0.40^{A}	16.18±0.84^{c}
BHT*	73.87±0.26^{D}	73.87±0.26^{d}

(A – D) Means within the same sample (cell-free supernatants). (a – d) Means within the same sample (intracellular cell-free extracts) above the bars are significantly different at $p<0.05$, as observed by Duncan's multiple range test. The data were expressed as mean ± SD.
*BHT concentration: 100 μg/ml.

Lipid peroxidation inhibition activity

Unsaturated fatty acids are oxidized to produce lipid peroxides. The onset of lipid peroxidation is the major initial product of lipid oxidation with lipid autooxidation, which plays a vital role in the oxidative degradation of lipids. It is the primary product of lipid oxidation and toxic and can damage DNA. Malondialdehyde is a secondary product of lipid peroxidation and has a high reactivity. It causes biomolecule damage of protein and DNA. Upon exposure to malondialdehyde, the cell morphology and protein synthesis ability decrease. Therefore, the control of lipid peroxides in the body is very important. Table 5 shows the inhibition of lipid peroxidation by lactic acid bacteria. All lactic acid bacteria showed the ability to inhibit lipid peroxidation to a different extent. The inhibition rates of cell-free supernatants were significantly higher than intracellular cell-free extracts. The cell-free supernatants, except for YMT1, were more than 50%, and BZ4 and SH14 had the highest values of 55.29±1.16% and 55.07±0.87%, respectively. Cell-free extracts showed more than 20% inhibition of lipid peroxidation in the intracellular cell-free extracts, except for YMT1. Lactic acid bac-

Table 5. Inhibition rate of lipid peroxidation of selected lactic acid bacteria

Strain	Inhibition rate of lipid peroxidation (%)	
	Cell-free supernatants	Intracellular cell-free extracts
BZ4	55.29±1.16^{D}	22.95±0.35^{b}
SH1	50.33±0.76^{B}	27.77±1.50^{d}
SH14	55.07±0.87^{D}	24.95±0.85^{c}
YMT1	43.83±1.32^{A}	11.18±0.69^{a}
YMT6	52.53±0.83^{C}	24.94±0.69^{c}
BHT*	93.69±0.33^{E}	93.69±0.33^{e}

(A – E) Means within the same sample (cell-free supernatants), (a – e) Means within the same sample (intracellular cell-free extracts) above the bars are significantly different at $p<0.05$, as observed by Duncan's multiple range test. The data were expressed as mean ± SD.
*BHT concentration: 100 μg/ml.

Table 6. Antimicrobial activities of selected lactic acid bacteria

Indicator strains	Inhibition zone (mm)			
	C. albicans ATCC10231	*E. coli* KCTC2571	*H. pylori* HPKCTCB0150	*L. monocytogenes* KCTC13064
Lactic acid bacteria				
BZ4	ND	20.3±0.42^{f}	21.5±0.46^{d}	19.4±0.17^{e}
SH1	ND	18.3±0.31^{d}	19.5±0.25^{c}	18.4±0.36^{d}
SH14	ND	16.2±0.15^{b}	17.3±0.20^{b}	15.3±0.30^{a}
YMT1	ND	15.3±0.21^{a}	17.4±0.42^{b}	16.3±0.17^{b}
YMT6	ND	16.5±0.15^{c}	14.0±0.30^{a}	17.2±0.25^{c}
Antibiotics				
Ampicillin*	ND	19.7±0.25^{e}	31.9±0.40^{e}	34.0±0.20^{f}
Amphotericin B†	11.9±0.40	ND	ND	ND

Means with differed letters (a - f) placed alongside values in the same pathogenic strains are significant at $p<0.05$, as confirmed by Duncan's multiple range tests.
ND: Not detected.
*Ampicillin concentration: 10 μg/ml.
†Amphotericin B concentration: 50 μg/ml.

teria have been reported to be regarded as antioxidant strains if the lipid peroxidation inhibition rate is above 20%. This observation suggests that lactic acid bacteria can inhibit lipid peroxidation.

Antimicrobial and antifungal activity

This study aimed to investigate the inhibitory activity against bacterial and fungi that cause food poisoning and various diseases. The results of the experiment using each of *C. albicans* ATCC 10231, *E. coli* KCTC 2571, *H. pylori* HPKCTC B0150, *L. monocytogenes* KCTC 13064, and *S. aureus* KCTC 1916 as an indicator strain are as follows (Table 6). As a control, MRS broth without lactobacillus was used, and no inhibition zone was observed. Regardless of the difference in the cell wall, the isolated lactic acid bacteria showed antimicrobial activity against Gram-positive and negative bacteria. However, *C. albicans* ATCC 10231 did not show an inhibition zone. BZ4 showed a high antimicrobial activity against all pathogenic strains. In particular, *E. coli* KCTC 2571 showed a higher inhibitory activity than Ampicillin 10 μg/ml, an antimicrobial agent. The antimicrobial activity against fungi was not observed; however, the antimicrobial activity against the other four indicator strain was confirmed. One of the major mechanisms of expression of antifungal susceptibility in candida infection is known as biomembrane. The resistance mechanisms of biomembrane protect fungi and increase resistance to antifungal agents. As a result, this process has been reported to cause antifungal agent treatment failure. Therefore, lactic acid bacteria could not be inhibited for this reason. Lactic acid bacteria are known to inhibit the growth of pathogenic bacteria and spoilage bacteria by organic acids, lower fatty acids, hydrogen peroxide, diacetyl, and bacteriocin. Therefore, we confirmed the possibility of lactic acid bacteria as a viable agent to replace antibiotics.

Identification of lactic acid bacteria

Before molecular biology identification, staining with a gram staining kit was performed to increase the efficiency of primer usage and the staining results and the morphology of the strains were observed with an optical microscope. All lactic acid bacteria were Gram-positive. BZ4, YMT1, and YMT6 appeared to be cocci-shaped, whereas SH1 and SH14 appeared to be rod-shaped. After confirming the morphological characteristics, we performed 16S rDNA sequencing. In addition, the availability of 49 carbon sources was confirmed by Api 50 CHL kit and the proximity to the standard species was read via the API web program. As a result, BZ4 was identified in *Pediococcus inopinatus* (probability of 99.9%), SH1 is *Lactobacillus plantarum* (probability of 99.7%), SH14 is *Lactobacillus brevis* (probability of 97.2%), YMT1 is *Pediococcus pentosaceus* (possibility of 98.2%) and YMT6 is *Leuconostoc mesenteroides* (probability of 98.1%). This is the same as the result of 16S rDNA sequencing.

Discussion

Probiotics are viable, non-pathogenic microorganisms that

can reach the intestine in sufficient numbers to provide health benefits to the host when ingested [8]. Probiotic intake promotes the growth of beneficial microorganisms and reduces pathogens to improve the host's gut microbial balance and gastrointestinal disease [3, 19]. Most probiotic bacteria are lactic acid bacteria and are generally isolated from fermented foods containing sufficient amounts of viable and active microorganisms [7]. Therefore, we separated lactic acid bacteria from Korean traditional fermented foods to find out the value of their use as probiotics. Lactic acid bacteria were isolated from some Korean traditional fermented food such as sikhae and young radish kimchi and bean-curd dregs. To select strains that can be used as probiotics in the isolated lactic acid bacteria, experiments on acid and bile salt tolerance were carried out by using artificial gastric juice and bile similar to the intestinal environment of the human body. As a result, five strains with high survival rates were selected. The selected strains were proved to be useful as probiotics by demonstrating appropriate cell surface characteristics along with antioxidant and antimicrobial properties. BZ4, SH1, SH14, YMT1, and YMT6 were identified as *P. inopinatus*, *L. plantarum*, *L. brevis*, *P. pentosaceus*, and *Lc. mesenteroides,* respectively. All five strains showed superior probiotics characteristics and various physiological activities, which are considered as valuable to be used a probiotic. In contrast to the previous studies on probiotics by separating lactic acid bacteria from kimchi, our study explored the probiotic effect on lactic acid bacteria isolated from the sikhae, one of the traditional fisheries fermented food and bean-curd dregs as a by-product of tofu production. Therefore, this study is expected to be used as a basic data for the search for new lactic acid bacteria and its potential as probiotics.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Brand-Williams, W., Cuvelier, M. E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol*. **28**, 25-30.
2. Chang, Y. H., Kim, J. K., Kim, H. J., Yoon, J. H., Kim, W. Y., Choi, Y. W., Lee, W. J., Kim, Y. B. and Park, Y. H. 1999. Characteristics of *Lactobacillus reuterii* BSA-131 isolated from swine intestine. *J. Kor. Appl. Microbiol. Biotechnol*. **27**, 23-27.
3. Chiang, S. S. and Pan, T. M. Beneficial effects of Lactobacillus paracasei subsp. paracasei NTU 101 and its fermented products. *Appl. Microbiol. Biotechnol*. **93**, 903-916.
4. Cho, K. M., Math, R. K., Hong, S. Y., AsrafulIslama, S. M., Mandannaa, D. K., Cho, J. J., Yun, M. G., Kim, J. M. and Yun, H. D. 2009. Iturin produced by *Bacillus pumilus* HY1 from Korea soybean sauce (kanjang) inhibits growth of aflatoxin producing fungi. *Food Cont*. **20**, 402-406.
5. Clements, A., Young, J. C., Constantinou, N. and Frankel, G. 2012. Infection strategies of enteric pathogenic *Escherichia coli*. *Gut Microbes*. **3**, 71-87.
6. Collado, M. C., Isolauri, E., Salminen, S. and Sanz, Y. 2009. The impact of probiotic on gut health. *Curr. Drug Metab*. **10**, 68-78.
7. Cross, M. L. 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunol. Med. Microbiol*. **34**, 245-53.
8. De-Vrese, M. and Schrezenmeir, J. 2008. Probiotics, prebiotics, and synbiotics. *Adv. Biochem. Eng. Biotechnol.* **111**, 1-66.
9. Douglas, L. J. 2003. *Candida* biofilms and their role in infection. *Trends Microbiol*. **11**, 30-36.
10. Doyle, R. J. and Rosenberg, M. 1995. Measurement of microbial adhesion to hydrophobic substrates. *Methods Enzymol*. **253**, 542-550.
11. Enomoto, H., Watanabe, H., Nishikura, K., Umezawa, H. and Asakura, H. 1998. Topographic distribution of *Helicobacter pylori* in the resected stomach. *Eur. J. Gastroenterol. Hepatol*. **10**, 473-478.
12. Farber, J. M., Sanders, G. W., Dunfield, S. and Prescott, R. 1989. The effect of various acidulants on the growth of *Listeria monocytogenes. Lett. Appl. Microbiol*. **9**, 181-183.
13. Fukushima, Y., Kawata, Y., Hara, H., Terada, A. and Mitsuoka, T. 1998. Effect of a probiotic formula on intestinal immunoglobulin a production in healthy children. *Int. J. Food Microbiol*. **30**, 39-44.
14. García-Ruiz, A., González de Llano, D., Esteban-Fernández, A., Requena, T., Bartolomé, B. and Victoria Moreno-Arribas, M. 2014. Assessment of probiotic properties in lactic acid bacteria isolated from wine. *Food Microbiol*. **44**, 220-225.
15. Javadi, A., Shamaei, M., Ziazi, L. M., Pourabdollah, M., Dorudinia, A., Seyedmehdi, S. M. and Karimi1, S. 2014. Qualification study of two genomic DNA extraction methods in different clinical samples. *Tanaffos* **13**, 41-47.
16. Kim, H. S. and Ham, J. S. 2003. Antioxidative ability of lactic acid bacteria. *Kor. J. Food Sci. Ani. Resour*. **23**, 186-192.
17. Kong, B. H., Zhang, H. Y. and Xiong, Y. L. 2010. Antioxidant activity of spice extracts in a liposome system and in cooked pork patties and the possible mode of action. *Meat Sci*. **85**, 772-778.
18. Ozlem, O., Fadime, K. and Ingolf, F. N. 2011. A probiotic bacterium, *Pediococcus pentosaceus* OZF, isolated from human breast milk produces pediocin AcH/PA-1. *Afr. J. Food Sci*. **10**, 2070-2079.
19. Oliveira M. N., Sodini, I., Remeuf, F. and Corrieu, G. 2001. Effect of milk supplementation and culture composition on

acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. *Int. Dairy J.* **11**, 11-12.
20. Pellegrin, N., Roberta, R., Min, Y. and Catherine, R. E. 1998. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay. *Method. Enzymol.* **299**, 379-389.
21. Petri, A., Pfannebecker, J., frohlich, J. and Konig, H. 2013. Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiol.* **33**, 48-54.
22. Raouf, T., Marion, B., Marielle, G. and Jean-Paul, V. 2013. *In vitro* characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic lactobacillus strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. *J. Med. Microbiol.* **62**, 637-649.
23. Sybesma, W., Hugenholtz, J., de Vos, W. M. and Smid, E. J. 2006. Safe use of genetically modified lactic acid bacteria in food, bridging the gap between consumers, green groups, and industry. *Electron. J. Biotechnol.* **9**, 424-448.
24. Tokatlı, M., Gülgör, G., BağderElmacı, S., Arslankozİşleyen, N. and Özçelik, F. 2015. *In vitro* properties of potential probiotic indigenous lactic acid bacteria originating from traditional pickles. *Biomed. Res. Int.* **2015,** 315819.
25. Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L. and Fowler, V. G. Jr. 2015. *Staphylococcus aureus* infections: *epidemiology*, *pathophysiology*, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603-661.
26. Vrese, M., Stegelmann, A., Ritcher, B., Fenselau, S., Laue, C. and Schrezenmeir, J. 2011. Probiotics: compensation for lactase insufficiency. *Am. J. Clin. Nutr.* **73**, 421-429.

초록 : 한국 전통발효식품에서 분리한 유산균의 프로바이오틱스 특성 및 건강기능성 연구

온정은[1] · 설민경[1] · 배은영[1] · 조영제[1] · 정희영[2] · 김병오[1]*
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본 연구에서는 한국의 전통발효식품인 식해, 열무김치, 비지에서 분리한 유산균의 프로바이오틱스로써의 사용 가능성을 확인하였다. 분리된 유산균 중 pH 2.5의 산에서 60% 이상의 생존율을 나타내는 *Pediococcus inopinatus* BZ4, *Lactobacillus plantarum* SH1, *Lactobacillus brevis* SH14, *Pediococcus pentosaceus* YMT1, *Leuconostoc mesenteroides* YMT2는 0.3% 담즙산에서도 모두 우수한 생존율을 나타내어 이 5종을 선별하여 실험을 진행하였다. 간접적으로 유용미생물의 군집화 및 병원성 세균의 부착을 저해하는 자가 응집 및 상호 응집 실험에서 다섯 개의 유산균은 강력한 응집능을 나타내었다. 유기용매를 이용한 세포 표면 소수성 실험에서 3가지 용매에 모두 부착성을 나타내어 세포 표면의 높은 소수성을 보여주었으며 이는 간접적으로 장세포에 부착할 수 있는 세포 부착능이 우수하다는 것을 보여준다. 또한, DPPH, ABTS 라디컬 소거능 측정, 지질 과산화억제능 실험에서도 선별된 유산균의 cell-free supernatant 및 intracellular cell-free extract는 항산화 활성을 나타내었다. 마지막으로 진균인 *C. albicans* ATCC 10231를 제외한 4가지 병원성세균(*E. coli* KCTC 2571, *H. pylori* HPKCTC B0150, *L. monocytogenes* KCTC 13064, *S. aureus* KCTC 1916)에서 모두 항균활성이 나타남을 확인하였다. 상기 실험결과를 바탕으로, 분리된 유산균은 항산화, 항균활성을 보유하고 있는 프로바이오틱스 제제로써 활용이 가능할 것으로 기대되며 이는 기초적인 실험으로써 산업화를 위한 임상검증 등의 추가적인 연구가 필요하다고 사료된다.