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Candidate of Probiotic Bacteria Isolated from Several *Jeotgals*: Korean Traditional Fermented Seafoods

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Abstract

Seventy eight bacterial strains were isolated from several *jeotgals* using MRS and M 17 agar media. The probiotic properties such as tolerance of extreme growth condition, production of antimicrobial compound, production of hydrogen peroxide, and enzymatic activity of bile salt hydrolase were investigated. DHK 4, 10, 21 and 74 strains showed a strong tolerance property against extreme conditions such as low pH and 0.5% oxgall-supplemented medium. DHK 10 and 47 strains produced hydrogen peroxide on TMB agar plate. DHK 8 and 10 strains produced antimicrobial compounds onto MRS agar against *E. facalis*. DHK 4, 6, 21, 29, 33, 63 and 87 strains had high activities of bile salt hydrolase. Especially, DHK 10 displayed a strong probiotic candidate; the abilities to produce the antimicrobial compound, hydrogen peroxide, and bile salt hydrolase. All these strains are assumed to be useful probiotic candidates. Among 78, twenty seven strains which have probiotic properties were tentatively identified by 16S rRNA sequencing. Among them, 7 *Lactobacillus* spp., 6 *Leuconosotoc* spp., 2 *Weisella* spp., 1 *Pediococcus* sp., 1 *Staphylococcus* sp., 1 *Enterococcus* sp. and 2 *Streptococcus* spp. were tentatively identified.

Key words: probiotic, lactic acid bacteria, bile salt hydrolase, hydrogen peroxide, 16S rRNA sequencing, *jeotgal*

INTRODUCTION

The fermentation of traditional fermented foods is frequently caused by natural, wild-type lactic acid bacteria (LAB) that originated from the raw material or the environment. There are a lot of traditional fermented foods in Korea, especially *jeotgal* made of seafood, and *jeotgal* includes more salt than other Korean fermented foods. It was used as the source of Kimchi and many different types of *jeotgals* exist depending on the materials used. During the fermentation of *jeotgal*, LAB is improving taste and flavor.

Probiotic bacteria are beneficial effects to the consumers (1). Probiotics prevent pathogen bacteria through the production of antimicrobial compound, lactic acid, hydrogen peroxide etc. (2). Several investigators have suggested another probiotic property that certain LAB are capable of reducing cholesterol for human health (3). Also, the probiotic bacteria survived in gastro intestinal track (GIT) passing through stomach and duodenums (3). According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group, two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies (4).

The objective of this work was to isolate the strains from several *jeotgals*, Korean traditional fermented seafoods and to investigate potential probiotics *in vitro* studies. The probiotic properties such as bile salt tolerance, acid resistance, production of antimicrobial substance, bile salt hydrolase activity and production of hydrogen peroxide were investigated from the isolated strains.

MATERIALS AND METHODS

Sample collection

Jeotgal samples were purchased from local grocery store at Jukdo market, Jukdo-dong, Pohang, Gyeongbuk province, Korea. The original sources of *jeotgals* used are squid, internals of Alaska Pollack, fermented Pacific saury (Kwamegi), codfish gill, octopus and shrimp. All samples were kept aseptically in sterile poly-bags with an ice-box, and transported to the laboratory. All samples were kept at 4~8°C until use.

Isolation of bacterial strains

For isolation of bacterial strains from *jeotgal*, 1 g of *jeotgal* sample was homogenized with 0.9% (w/v) sterile physiological saline and further diluted in a ten-fold serial

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dilution with the same solution. One hundred microliters of the appropriated dilution were spread onto de Man, Rogosa and Sharpe (MRS) and M 17 agar plates (pH 6.5). All plates were incubated at 30°C for 48 hr. The colonies were selected randomly and purified by re-streaking. The purified strains were kept at -80°C in MRS broth (Merck) containing 20% (v/v) glycerol (5) until use.

Phenotypic characterization

Cell morphology, arrangement and gram-stain were characterized by microscopy (6). Also catalase test was carried out using 0.3% (v/v) H_2O_2 (Junsei, Japan) solution. The accumulation of CO_2 gas in Durham tube within MRS broth is attributed to production from glucose.

Bacterial growth was observed at different conditions (temperatures 10°C, 45°C/pH 3.9, pH 9.6/6.5% NaCl) according to Schillinger and Lücke (7) and Stiles and Holzapfel (8).

The experiment for production of ammonia from arginine was carried out by the procedures of Harrigan and McCance (9).

The configuration of D and L isomers of lactic acid produced from glucose was determined by an enzymatic method. D- and L-lactate dehydrogenase test using commercial kit (Hoffman La Roche Diagnostic Mannheim, Germany) was done.

The presence of *meso*-diaminopimelic acid (DAP) in bacterial cell wall was determined using thin-layer chromatography on a cellulose plate by Marconi et al. (10).

Low pH tolerance and bile salt tolerance

For the tolerance at low pH, the isolates were incubated in a MRS broth containing 0.05% (w/v) L-cysteine · HCl · H_2O (Junsei, Japan) at 30°C for 24 hr. The MRS broth was adjusted to pH 2.0 by using 1 N HCl (Junsei, Japan). The isolates were inoculated into the MRS broth (pH 2.0), then incubated at 30°C for 24 hr. To determine low pH tolerance, broth culture medium was measured at 600 nm by using UV spectrophotometer. The broth of non-inoculated low pH sample was used as OD control value (11). To increase accuracy, the broth was checked three different samples in the same condition and made average values.

To access bile salt tolerance, 0.5% (w/v) oxgall (Difco, Detroit, USA) was supplemented in MRS broth. All bacteria were incubated at 30°C for 24 hr. The entire inoculated samples were checked by measuring OD value three times and made average values (12).

Bile salt hydrolase (BSH) activity

In order to assess BSH enzyme of bacteria, the bacterial strains isolated from *jeotgal* were incubated in normal broth at 30°C for 24 hr. Thirty microliters of the cultured broth were impregnated around sterilized paper disk on MRS agar plate supplemented with 0.5% (w/v) sodium salt of taruodeoxycholic acid (TDCA) and 0.37 g/L $CaCl_2$. Plates were anaerobically incubated at 30°C for 72 hr, and the diameter of the precipitation zones around the disks was measured (13).

Production of hydrogen peroxide

The qualitative determination of the H_2O_2 produced by the strains was demonstrated using a qualitative method modified by Liliana et al. (14). One percent of bacterial suspension of the isolated strains was inoculated onto a MRS agar plate with 0.25 mg/mL of 3,3′5,5′-tetramethylbenzidine (TMB); 0.01 mg/mL horseradish peroxidase generates O_2 from H_2O_2 produced by LAB, and TMB dyes the colonies with a blue color when oxidation occurs in the presence of O_2. After 48 hr incubation at 30°C in anaerobic jar, the colonies that produce H_2O_2 turns to a blue color.

The test of preliminary antimicrobial activity

In order to determine preliminary antimicrobial activity, the bacterial strains were grown in MRS broth at 30°C for 24 hr. Four microliters of the culture were spotted onto MRS agar, incubated at 30°C for 24 hr and then overlaid with active growing cells of the target strain, imbedded in MRS or LB medium (0.8% agar, w/v). One percent of target strain was inoculated in 9 mL soft agar. The plates were incubated at 37°C for 24 hr and the colonies were examined for the formation of inhibition zones. All experiments were replicated three times (15).

16S rRNA sequencing

PCR amplification of the 16S rDNA was performed by PCR using two universal primers (27F and 1492R). The PCR product was purified by using Solgent PCR purification kit (cat no. SGP 2101, Solgent company, Korea). The purified 16S rDNA was sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) as recommended by the manufacturer. The purified sequencing reaction mixtures were automatically electrophoresed using ABI Prism 3730x1 DNA analyzer.

RESULTS

Phenotypic characterization

Seventy eight strains were isolated using MRS and M 17 agar media from several *jeotgal* samples. Fifty-eight strains were isolated from MRS agar medium, 20 from M 17. All the strains were identified to phenotypic

Table 1. Phenotypic characteristics of the strains isolated from *jeotgal*

Isolated no.	Cell-morphology	D.L lactate	CO_2/ glucose	Catalase	Gram staining	10°C	45°C	pH 3.9	6.5% NaCl	Arginine
DHK 1	ovoid	L	-	-	+	+	+	+	+	+
DHK 2	ovoid pair	L	-	-	+	+	-	+	+	+
DHK 4	rod	DL	+	-	+	+	+	+	+	-
DHK 6	ovoid pair	DL	+	-	+	+	-	+	+	-
DHK 7	ovoid chain	L	-	-	+	+	+	+	+	-
DHK 10	short rod chain	DL	-	-	+	+	+	+	+	-
DHK 14	rod chain	DL	-	-	+	+	-	+	+	-
DHK 16	short rod, ovoid	DL	+	-	+	+	-	+	+	-
DHK 18	ovoid chain	L	-	-	+	+	+	+	+	-
DHK 19	ovoid chain	DL	+	-	+	+	+	+	+	-
DHK 21	short rod chain	DL	+	-	+	+	+	+	+	-
DHK 22	rod	DL	-	-	+	+	+	+	+	-
DHK 25	ovoid pair, quarter	DL	-	-	+	+	+	+	+	-
DHK 29	rod	L	-	-	+	+	+	+	+	-
DHK 33	ovoid pair	DL	+	-	+	+	-	+	+	-
DHK 36	ovoid pair	L	-	-	+	-	-	-	+	-
DHK 38	ovoid quarter	DL	-	-	+	+	-	-	+	-
DHK 39	ovoid pair, quarter	DL	-	-	+	-	-	-	+	+
DHK 55	ovoid chain	DL	+	-	+	+	+	+	+	-
DHK 58	ovoid chain	DL	+	-	+	+	+	+	+	+
DHK 60	ovoid chain	DL	+	-	+	+	+	+	+	-
DHK 63	ovoid chain	L	-	-	+	+	+	-	+	+
DHK 67	ovoid chain	L	-	-	+	+	+	+	+	+
DHK 71	ovoid chain	L	-	-	+	-	+	+	+	-
DHK 72	ovoid chain	L	-	-	+	-	-	+	+	-
DHK 73	ovoid chain	DL	-	-	+	-	-	-	+	+
DHK 74	ovoid chain	L	-	-	+	-	+	+	+	-

+: grown, -: not-grown.

levels such as cell morphology by microscope, gas production from glucose, growth behavior at 10°C, 45°C, growth in 6.5% (w/v) NaCl and growth in pH 3.9. Twenty seven strains were gram-positive and catalase negative (Table 1).

Low pH tolerance, bile salt tolerance and bile salt hydrolase activity

Low pH tolerance of the selected strains in Table 1 was assessed in pH 2.0 and 0.5% (w/v) oxgall-supplemented MRS in order to identify the tolerance of 0.5% bile salt. Most strains tested were tolerable to bile salt and sixteen strains had tolerance in pH 2.0 (Table 2). Four strains including DHK 4, 10, 21 and 74 showed higher surviving rate than other strains (Table 2).

The cell numbers in these strains increased in extreme condition such as pH 2.0 during 24 hr. The real stomach juices contain many enzymes and many ions. Most bacteria cannot survive in low pH. However, our probiotic candidate strains survived under extreme condition and they proliferated by 1×10^8/mL numbers after 24 hr incubation (data not shown).

Also the activity of bile salt hydrolase was measured by MRS plate containing taurodeoxycholic acid and $CaCl_2$. Among 19 strains which produced BSH on the agar plate, 7 strains containing DHK 4, 6, 21, 39, 33, 63 and 87 showed stronger activities of BSH (Table 3). The results show us that these strains might be possible candidates to decrease cholesterol level in our body.

Production of hydrogen peroxide and preliminary antimicrobial effect

The production of hydrogen peroxide was detected by TMB agar plate. DHK 10 and 47 strains produced H_2O_2 qualitatively on TMB agar (data not shown). These two strains produced H_2O_2 in anaerobic condition and they affected the preservation period *of jeotgal*. DHK 10 was tentatively identified as *Lactobacillus plantarum*, but DHK 47 was not identified yet (Table 4).

Moreover we used the spot and lawn method to test antimicrobial effect of the isolated strains. The indicator microorganism, *E. feacalis* was used. The clear zones appeared in many strains on MRS agar plate (data not shown). Especially, DHK 8 and 10 exhibited the strongest activity against indicator microorganism (Fig. 1).

Tentative identification by 16S rRNA sequencing

Among 78 isolated, twenty seven strains which had probiotic properties were tentatively identified by 16S rRNA sequencing. Among them, 7 *Lactobacillus* spp.,

Table 2. Growth check in 0.5% oxgall-supplemented MRS and pH 2.0

Isolated no.	*Jeotgal* source	Tentative identification	OD value in pH 2.0	Growth in 0.5% oxgall (w/v)
DHK 1	Squid	*Pediocuccus pentosaceus*	-	+
DHK 2	Squid	*Enterococcus lactis*	-	+
DHK 4	Squid	*Leuconostoc paramesenteroides*	***	+
DHK 6	Squid	*Leuconostoc citreum*	-	+
DHK 7	Squid	*Lactobacillus plantarum*	*	+
DHK 10	Squid	*Lactobacillus plantarum*	***	+
DHK 14	Squid	*Lactobacillus plantarum*	*	+
DHK 16	Squid	*Leuconostoc paramesenteroides*	**	+
DHK 18	Squid	*Lactobacillus plantarum*	*	+
DHK 19	Squid	*Leuconostoc citreum*	**	+
DHK 21	Squid	*Lactobacillus plantarum*	***	+
DHK 22	Squid	*Lactobacillus plantarum*	**	+
DHK 25	Alaska Pollack internals	Not identified	-	+
DHK 29	Fermented Pacific saury (Kwamegi)	Not identified	-	+
DHK 33	Squid	*Leuconostoc citreum*	*	+
DHK 36	Squid	Not identified	-	+
DHK 38	Fermented Pacific saury (Kwamegi)	Not identified	*	+
DHK 39	Squid	Not identified	-	+
DHK 55	Codfish gill	*Weissella paramesenteroides*	*	+
DHK 58	Octopus	*Leuconostoc citreum*	-	+
DHK 60	Octopus	*Weissella paramesenteroides*	*	+
DHK 63	Octopus	*Staphylococcus pasterui*	**	+
DHK 67	Octopus	*Lactobacillus sakei*	*	+
DHK 71	Shrimp	*Streptococcus salivarius*	-	-
DHK 72	Shrimp	*Streptococcus salivarius*	-	+
DHK 73	Octopus	Not identified	-	+
DHK 74	Shrimp	Not identified	***	+

***: OD value>1.00 after 24 hr in 30°C incubator, **: 1.00>OD value>0.50 after 24 hr, *: 0.50>OD value>0.10 after 24 hr. +: grown, -: not-grown.

Most of probiotic candidate strains survived in 0.5% oxgall-supplemented MRS. OD values of DHK 4, 10, 21, and 74 strains in pH 2.0 were more than 1.00. It means that these four strains had tolerance in low pH condition and the strains might be the possibility to survive in human stomach.

Table 3. Bile salt hydrolase activity on MRS agar plate containing taurodeoxycholic acid

Isolated no.	Tentative identification	Size of circle (cm)
DHK 4	*Leuconostoc paramesenteroides*	1.2
DHK 6	*Leuconostoc citreum*	1.3
DHK 21	*Lactobacillus plantarum*	1.35
DHK 29	Not identified	1.2
DHK 33	*Leuconostoc citreum*	1.2
DHK 63	*Staphylococcus pasterui*	1.15
DHK 87	Not identified	1.25

All strains were tested three times and then made average value. The above 7 strains had strong BSH activity.

Table 4. The production of hydrogen peroxide

Isolated no.	Tentative identification	H_2O_2 production
DHK 10	*Lactobacillus plantarum*	O
DHK 47	Not identified	O

Qualitative detection of hydrogen peroxide in TMB agar plate. O: Blue color in TMB agar plate.

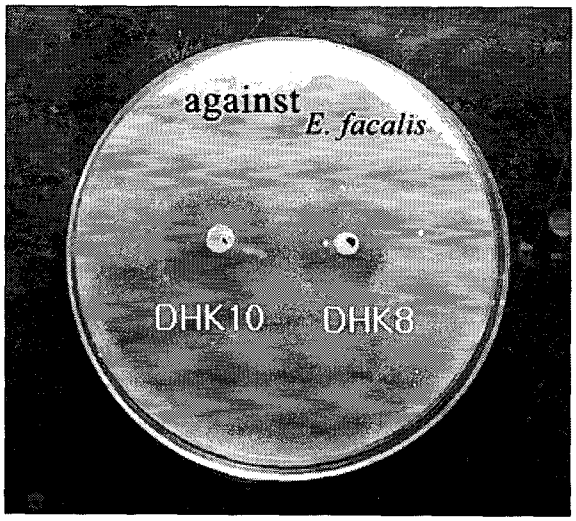

Fig. 1. Test of preliminary antimicrobial effect by spot and lawn method.

E. facalis was used as indicator strain and 3 μL from each strain were spotted on MRS agar plate. This plate was incubated at 37°C for 48 hr. The clear zones were measured.

6 *Leuconosotoc* spp., 2 *Weisella* spp., 1 *Pediococcus* sp., 1 *Staphylococcus* sp., 1 *Enterococcus* sp. and 2 *Streptococcus* spp. were identified, except seven (Table 2).

DISCUSSION

Lactic acid bacteria are one of the most popular strains for probiotics. *Leuconostoc* spp. and *Lactobacillus* spp. were 13 among 20 strains in our present study (Table 2). In previous studies, there are reports on LAB isolated from fermented milk, dairy food (5). Moreover, the diverse probiotic properties such as production of antimicrobial compound (15), reduction of cholesterol level (3), enhancement of specific immune response (16), and increasing safety of normal flora in intestinal track were detected from fermented foods.

Our results suggest that *jeotgal* is good candidate as functional food. The results showed that a lot of LAB coacted in the fermentation of *jeotgal*. The various properties were influenced by LAB during fermentation. Especially, these results revealed the possibility of probiotic bacteria isolated from *jeotgal*. We conclude that the antimicrobial compounds produced by LAB and H_2O_2 improved preservation period through the inhibition of pathogen. Our results have shown a drastic inhibition against *E. fecalis* by spot and lawn method. In future, the antimicrobial compounds will be elucidated from the probiotic candidates shown above.

The bile salt hydrolase activity of LAB has been well documented (12,13). Based on the data obtained in our study, several strains had BSH activity after 48 hr incubation. Our results suggested that the tested strains had the possibility to decrease cholesterol level in our body through the BSH working in GIT (12,13). Most LAB isolated from *jeotgal* survived under extreme conditions such as low pH (artificial gastric juice) and 0.5% (w/v) bile salt (artificial bile acid). Because of these properties, the strains isolated from *jeotgal* will be good survivor in our GIT, and also they are possible producer of antimicrobial compound against pathogen in GIT. The conclusion in the experiment is that DHK 10 strain is the best probiotic candidate.

Especially, DHK10 identified as *Lactobacillus plantarum* had strong antimicrobial activity, positive property of BSH production, tolerance of extreme growth condition, and also production of H_2O_2. Therefore, we will expect that this strain enhances our health in GIT and reduces cholesterol level in our body. Furthermore, we will test cytokine induction using human enterocyte cell line to investigate GIT immune response against probiotic strains.

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