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Isolation and Identification of Lactic Acid Bacteria Isolated from a Traditional Jeotgal Product in Korea

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Abstract – Seventeen lactic acid bacterial strains (LAB) were isolated using MRS agar medium from Jeotgal, a Korean fermented food, purchased at the Jukdo market of Pohang. To identify the strains isolated, they were tested by examining their cell morphologies, gram-staining, catalase activity, arginine hydrolase activity, D-L lactate form and carbohydrate fermentation. According to the phenotypic characteristics, three strains were tentatively identified as *Lactobacillus* spp., ten were *Enterococcus* spp. (or *Streptococcus* spp., or *Pediococcus* spp.) and the rest were *Leuconostoc* spp. (or *Weissella* spp.). Five strains among 17 were chosen by preliminary bacteriocin activity test. Four bacterial strains which inhibited both indicator microorganisms were identified by 16S rRNA sequencing. The results are as follows; *Leuconostoc mesenteroides* (HK 4), *Leuconostoc mesenteroides* (HK 5), *Leuconostoc mesenteroides*(HK 11), *Streptococcus salivarius*(HK 8). In order to check LAB which are showing a high survival rate in gut, we investigated three strains inhibiting both indicator microorganisms in artificial gastric acid and bile juice –all except HK8. The three strains mentioned above grew in extreme low acid conditions.

Key words – Lactic acid bacteria, Jeotgal, Bacteriocin, 16S rRNA sequencing

1. Introduction

Korean fermented foods called Jeotgal are very traditional seafood products, which can be found in various East Asian countries with slightly different ingredients. In previous studies, there are reports on LAB isolated from fermented milk (Mathara *et al.* 2004), and production of antimicrobial compound (De Vuyst L *et al.* 2003). *Bacillus* spp. isolated from Jeotgal have been studied in detail. (Yoon *et al.* 2001).

On average, 5-10 g of the jeotgal are consumed per person weekly in Korea. Moreover it is the crude material for Kimchi. Jeotgal is made from raw seafood and fish by traditional methods. It contains more than 20% salt, whole seafood, and fish, including internal organs. Jeotgal is spontaneously fermented for at least two months.

Lactic acid bacteria (LAB) are well-known microorganisms in fermented foods like dairy products and processed vegetables. Some Lactic Acid Bacteria (LAB) were isolated from fish fermented food in Korea (Lee 1993). Moreover, a number of antimicrobial compounds were produced by LAB and these compounds are of great interest to the food industry because of their inhibitory activity against food spoilage or pathogenic microorganisms during food procession and food fermentation (Lee *et al.* 1999). However, lactic acid bacteria (LAB) isolated from regional jeotgal have not been studied well until now.

The aim of this work was to isolate lactic acid bacteria from regional jeotgal and characterize LAB which show inhibitory activity against indicator microorganisms. Also through this experiment, we examined the functional properties of the isolated strains, such as bile salt tolerance, acid resistances and antimicrobial activities.

2. Materials and Methods

Sample collection

Jeotgal samples were purchased from a local grocery store at Jukdo market, Jukdo-dong, Pohang, Kyungbuk

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province, Korea. 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.

Microbial enumeration and isolation

Ten grams of jeotgal samples were homogenized with 90 ml of 0.85% (w/v) sterile physiological saline in a Stomacher lab-blender(Stomacher 400, England) for 30 seconds and serially diluted (10^{-1}-10^{-8})(Mathara *et al.* 2004). One hundred microliters of appropriately diluted sample were spread on selective agar media. LAB were isolated on MRS (De Man, Rogosa and Sharpe, De Man *et al.* 1960) (Difco, Detroit, U.S.A) agar plates after incubation at 30°C for 48-72 hr. Also Violet Red Bile Dextrose Agar (VRBD) (Difco, Detroit, U.S.A) were used for the enumeration of *Enterobacteriaceae* (Mathara *et al.* 2004). Colonies were selected randomly and purified by re-streaking (Leisner *et al.* 1997). Purified strains of LAB were inoculated into MRS broth (pH 6.5) and incubated at 30°C for 24 hr. All purified strains were kept in MRS broth containing 20% glycerol at -70°C.

Phenotypic characterization

Cell morphology, arrangement and gram-stain were characterized by microscopy (Gerhardt *et al.* 1981). Also catalase test was carried out using 0.3% (v/v) H_2O_2 (Merck, Darmstadt, Germany) solution. CO_2 gas in Durham tube within MRS broth was checked from glucose. The growth of isolated LAB was tested at different conditions (temperatures; 10°C/45°C, different pHs and MRS within 6.5% NaCl concentration) according to the procedures described by Hammes and Vogel (1995), Schillinger and Lücke(1987), and Stiles and Holzapfel (1997).

The configuration of D(-) and L(+) isomers of lactic acid produced from glucose was determined by enzymatic method (Boehringer-Mannheim 1989). MRS broth without beef extract and acetate were used to assay lactic acid isomer. The commercial kit for D- and L- lactate dehydrogenase test (Hoffman La Roche Diagnostic Mannheim, Germany) was used for the identification (Boehringer-Mannheim 1989).

The presence of *meso*-DAP in the bacterial cell wall was determined using thin-layer chromatography on cellulose plates (Tamang *et al.* 2000). When the sample contains *meso*-DAP, the spot on TLC turns yellow color after several hours or days in the dark (Emanuels *et al.* 2000).

Carbohydrate fermentation patterns of LAB were determined using API CHL test strips (bioMërieux, France). For the assay of carbohydrate fermentation, the strains were grown at 30°C in MRS broth and the cells obtained by centrifugation at 10,000 rpm for 5 minutes. The pellets were washed 2 times with distilled water, then re-suspended in basal medium. The test kit was used according to the manufacturer's instructions. The result was interpreted using APILAB PLUS V3.2.2 software database.

The experiment for the production of ammonia from arginine was carried out according to the procedure described by Harrigan and McCance (1976). The MRS-arginine medium was described by Harrigan and McCance (1976).

Genotypic characterization

Chromosomal DNA was isolated by the modified method of Varmanen *et al.* (Varmanen *et al.* 1998). The DNA concentration was measured at 260 nm by using a UV spectrophotometer, and adjusted to 10 μg/ml by dilution with 10 mM Tris HCl and pH 8.0.

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.

Specific property

For the tolerance test at low pH, the isolates were incubated in a MRS broth containing L-cysteine·HCl·H_2O (Junsei, Japan) as 0.05% concentration (w/v) at 30°C for 24 hr. The MRS broth was adjusted pH to 2.5 by using 1N HCl (Junsei, Japan). The isolates were inoculated into the MRS broth (pH 2.5) as 30 μl volume, then incubated at 30°C for 3hr. Bacterial cultures were spread onto MRS agar plates and incubated at 30°C for 48 hr. Bacterial strains were confirmed to have low pH tolerance when the colonies were formed on MRS agar after 48 hours incubation (Kimoto *et al.* 1999).

0.5% (w/v) oxgall (Difco, Detroit, U.S.A) was supplemented with MRS broth. All bacterial strains inoculated were incubated at 30°C for 3 hr. Then, bacterial cultures were

spread onto the MRS agar plates to confirm the bile salt tolerance (Chung *et al.* 1997, De Smet *et al.* 1994, Gilland *et al.* 1984).

The LAB were inoculated into MRS broth adjusted to pH 6.5 before autoclaving and grown aerobically at 30°C. Acid production was determined by measuring pH of the cultures after 6 hr, 12 hr, 24 hr and 48 hr. MRS broths for all acid production test were prepared from a single batch which pH was adjusted and then dispensed into tubes of 10 ml each before autoclaving.

Preliminary bacteriocin activity test

Preliminary bacteriocin detection and activity were tested by a modification of the method of Ahn *et al.* (Ahn *et al.* 1990). The inhibitory potential of lactic acid bacterial cultures and their supernatants was investigated using the modified Agar Well Assay method as described by Shillinger and Lucke (1989). The MRS agar was poured into Petri dishes and left to solidify and dry for 1-2 days. Ten lactic acid bacterial strains isolated were cultured in MRS broth at 30 for 24 hr and 10 μl of the cultures transferred on MRS agar plate. The cultures inoculated on Agar plate were incubated at 30°C for 24 hr. 10 ml of soft agar (0.8%) were prepared by adding indicator microorganisms like *S. aureus* and *E. faecalis*, and were gently mixed and poured over the surface of pre-spot MRS agar plates. Activity was quantified by measuring diameter of clear zone per one spot.

3. Results

Phenotypic characteristics

Average mesophilic bacterial counts are 3.6×10^7 on PCA agar, and general LAB counts are 1.5×10^7 on MRS agar. No *Enterobacteriaceae* did appear on VRBD agar from the strains isolated (data not shown).

In total, 17 strains were isolated from regional Jeotgal and identified to genus level on the basis of their cell morphologies, gas production from glucose, growth behaviors at 10°C, 45°C, 6.5% NaCl, pH 9.6 and pH 3.9 according to Hammes and Vogel (1995). All strains tested were gram-positive and catalase-negative (Table 1). Three strains exhibited rod-shaped morphologies, produced DL lactate and did not produce CO_2 from glucose fermentation. Also the strains made DL lactate and had *meso*-DAP in the cell wall. Thus these were tentatively identified as *Lactobacillus* spp. Four strains showing coccoid morphologies, produced D-lactate and gas from glucose metabolism. They maybe belong to the genus *Leuconostoc* or *Weissella* spp. The other 10 strains were coccoid shapes, produced L lactate

Table 1. Clustering LAB by phenotypic characteristics

Cluster	Group A	Group B	Group C
Cell-morphology	rod	ovoid	ovoid
CO_2	-	-	+
Arginine	-	+ (4/10)	-
Bacteriocin	-	+ (2/10)	+ (3/4)
10°C	-	+ (4/10)	+
45°C	-	+ (2/10)	-
pH3.9	+	+	+
pH9.6	-	+ (9/10)	+ (3/4)
6.5% NaCl	-	+ (5/10)	+
Catalase	-	-	-
Gram stain	+	+	+
Strain no.(HK)	6,7,9	8,10,11,12,13,14,15,16,17,18	2,4,5,19
	Lactobacillus	*Enterococcus* or *Streptococcus* or *Pediococcus*	*Leuconostoc* or *Weissella*

Arginine hydrolysis; HK 10, 12, 14, 17 in Group B
Bacteriocin production; HK 8, 11 in Group B and HK 4, 5, 19 in Group C
Grown at 10°C; HK 8, 11, 16, 18 in Group B and all in Group C
Grown at 45°C; HK 14, 15 in Group B
Grown at pH 3.9; all in Group A, B, C
Not-grown at pH 9.6; HK 13 in Group B and HK 2 in Group C
Grown at 6.5% NaCl; HK 10, 11, 12, 13, 18 in Group B and all in Group C

Table 2. Inhibitory diameter by LAB

Bacterial No.	Against *S.aureus*	Against *E.faecalis*
HK4	2.2 cm	1.8 cm
HK5	1.7 cm	1.8 cm
HK8	0.6 cm	1.0 cm
HK11	1.2 cm	1.5 cm
HK19	None	1.8 cm

Plates are duplicated and then make the mean value.
HK4 was the strongest, among the strains inhibiting both indicators.
HK19 had inhibitory diameter only against *E.faecalis*.

Table 3. Carbohydrate utilization of the strains isolated from Jeotgal

Test	Reactions/Enzymes	HK5	HK11	HK4
LARA	L-Arabinose	+	+	+
MDX	β Methyl-D-xyloside	-	-	-
MAN	Mannitol	+	+	+
SOR	Sorbitol	-	-	-
MDG	α Methyl-D-glucoside	+	+	+
AMY	Amygdalin	+	-	+
CEL	Cellobiose	+	-	+
TRE	Trehalose	+	+	+
INU	Inulin	-	-	-
MLZ	Melezitose	-	-	-
RAF	Raffinose	+	+	+
AMD	Amidon	-	-	-
GEN	Gentiobiose	+	-	+
TUR	D-Turanose	+	+	+
GNT	Gluconate	+	-	+
2KG	2 keto-gluconate	+	+	+

+ : fermented -: not-fermented

or D L lactate, and did not produce gas from glucose fermentation. These strains were nearby genus of *Enterococcus* spp. or *Streptococcus* spp. or *Pediococcus* spp. (Table 1).

Table 4. Lactic acid bacteria identified using 16S rRNA sequencing

Bacterial no.	Identified as	Identities base/base (similarity %)
HK4	*Leuconostoc mesenteroides*	1428/1430 (99.86%)
HK5	*Leuconostoc mesenteroides*	1423/1424 (99.93%)
HK8	*Streptococcus salivarius*	1421/1423 (99.86%)
HK11	*Leuconostoc mesenteroides*	1359/1360 (99.92%)

Table 5. Low pH tolerance, bile salt tolerance on three strains isolated from Jeotgal

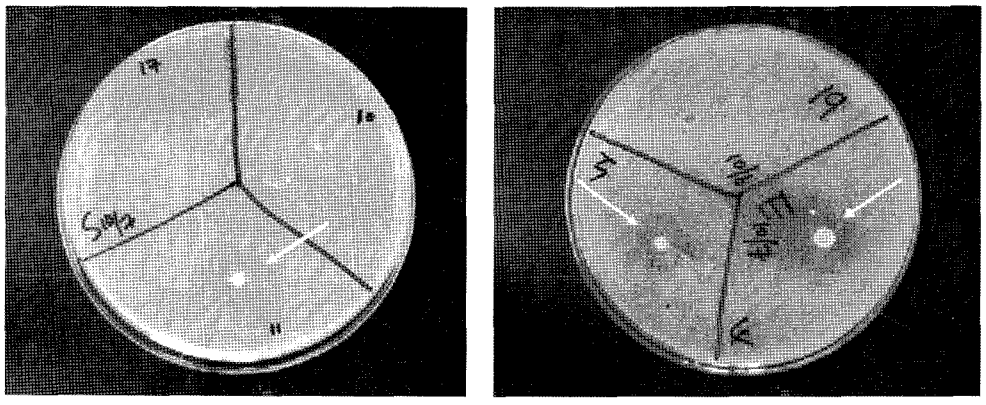
Strain no.	OD value in MRS (pH 2.5)	OD value MRS in 0.5% oxgall
HK4	0.241	0.213
HK5	0.21	0.2
HK11	0.214	0.191

Optical density (measured at 600 nm) was measured after 18 hrs. Delay of growth was observed. Only these three strains were grown in

As shown in Table 1, there are three groups; HK6,7,9 strains belong to Group A/HK8,10,11,12,13,14,15,16,17,18 strains are included in Group B/HK2,4,519 belong to Group C.

Any strains belonging to Group A did not grow in 10°C, 45°C, 6.5% NaCl and pH 9.6 except pH3.9. But several strains from Groups B and C grew in the above conditions (Table 1). Carbohydrate utilization was checked from HK 4, 5 and 11 strains with inhibitory activity (Table 3).

According to our experimental results in low pH tolerance and bile salt tolerance, several strains survived more than 3 hours (Table 5). Normal growth condition was checked by OD value. Figure 2 and Figure 3 show OD patterns dependent to time and the pH values. According to the results, pH decreased to 4.5 easily in 5 strains



(a) (b)

Fig. 1. Preliminary bacteriocin activity test. Bacteriocin activity was detected by the deferred inhibition assay. White arrow indicated inhibitory region. *S. aureus* and *E. faecalis* were used as indicator strains. (a): *E. faecalis* overlay, (b): *S. aureus* overlay.

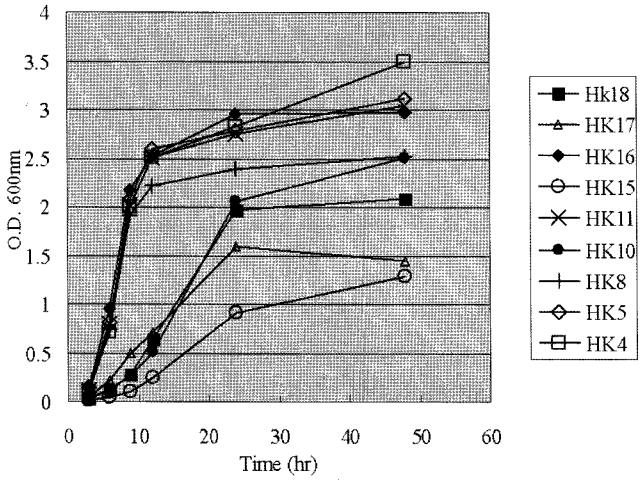


Fig. 2. Growth curve of lactic acid bacteria in MRS broth. Growth condition was checked by OD value in 600nm. After 50 hours, OD value did not change, because dead bacteria interfered OD value.

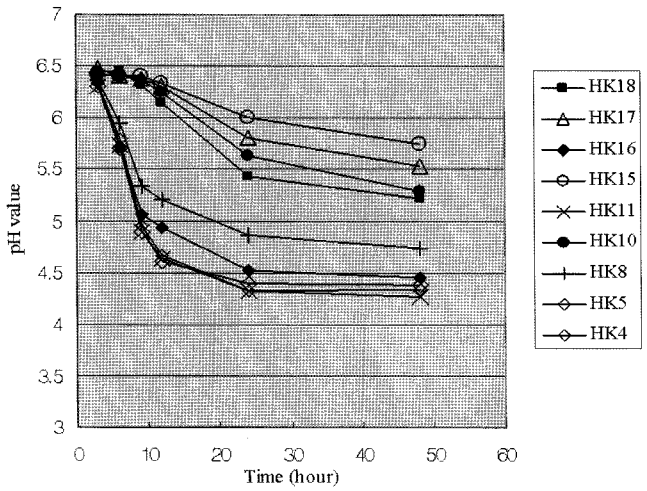


Fig. 3. pH decreases on growth time. pH decreased because LAB produced acid during growth in MRS broth.

during 24hr. pH decreased between 5.5 and 6, but pH changed after 3 days. OD value is indirect method to measure cell density. So after 48hr OD values did not decrease, OD value was maintained between 2.5 to 3. These results mean that dead cells affected to OD value when it was measured.

Preliminary bacteriocin activity

The inhibitory potential of lactic acid bacterial cultures in Jeotgal was investigated using two indicator microorganisms like *S. aureus* and *E. faecali*. Five strains (HK4,5,8,11,19) among 17 showed the inhibitory activities against indicator microorganisms (Table 2, Figure 1). HK 4 was the strongest among the strains inhibiting both indicators, but HK 19 had inhibitory activity only against *E. faecalis* (Table 2).

16S rRNA sequencing

Finally, four strains which had positive candidates from bacteriocin test were chosen for further identification using 16S rRNA sequencing. As shown in Table 4, there were *Leuconostoc mesenteroides* (HK 4), *Leuconostoc mesenteroides* (HK 5), *Leuconostoc mesenteroides* (HK 11) and *Streptococcus salivarius* (HK 8).

4. Discussion

We isolated bacteria using plate agar, VRBD agar, and MRS agar from Jeotgal, a Korean fermented food. Aerophilic bacteria were isolated by plate counting, but LAB were isolated using MRS agar. The strains isolated were clustered by physiological tests shown in Table 2. Three among seventeen strains were homofermented rod shapes, the other four ones were heterofermented coccoid and the rest were homofermented coccoid.

In our experiments we isolated several *Leuconostoc* spp. Therefore *Leuconostoc* spp. might be related to the source in Jeotgal fermentation. To check possible pathogenic bacteria, we plated on the possibilities of pathogens using VRBD agar, but no pathogens grew (data not shown). The possible reasons are as follows; 1) a quick drop in pH by LAB, 2) high salt concentration, 3) production of obstructive substances like hydrogen peroxide, 4) bacteriocin controlling growth of spoiling bacteria as a biopreservative (Stiles *et al.* 1997). Antimicrobial compounds are produced in various LAB (Klaenhammer 1988). Also bacteriocins can be used as biopreservatives in fermented foods (Ralph *et al.* 1995).

According to these results, 3 strains having rod shapes were *Lactobacillus* spp. and 10 homofermented coccoid shapes were *Enterococcus* spp. or *Streptococcus* spp. or *Pediococcus* spp. And the other heterofermented coccoid shapes were *Leuconostoc* spp or *Weissella* spp. Four bacterial strains showed the clear zones in two indicator microorganisms (Figure 1). The strains possessing bacteriocin activity isolated from Jeotgal were identified by 16S rRNA sequencing. Most were identified as *Leuconostoc* spp. The LAB isolated were roughly clustered by phenotypic

characteristics, the clustered groups (HK 4,5,8) were consistent with the results of 16S rRNA sequencing except HK 11 (Table 4).

The pH decreases very quickly when *Leuconostoc spp.* are dominant in jeotgal (Figure 3), so the biopreservative activity is increased. When these strains are dominant in jeotgal, the pH decreases very lowly and then it influences the texture, smell, and safety. As a result, Jeotgal texture is changed: soft and tender, in addition to improving food safety.

In further studies, we will test BSH secretion from LAB in Jeotgal (Begly M *et. al.* 2006). As reported on cholesterol lowering effect by lactic acid bacteria (Taranto MP *et al.* 2000) and bacteriocins (Todorov and Dicks, 2006), the LAB isolated from Jeotgal are being investigated as good candidates of probiotics.

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