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Physicochemical Properties and Bacterial Communities of Meongge (*Halocynthia roretzi*) Jeotgal Prepared with 3 Different Types of Salts

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Three types of meongge (*Halocynthia roretzi*) jeotgal (MJ) were prepared with 3 different types of salts (12%, w/v): purified salt (PS), solar salt aged for 3 years (SS), and bamboo salt that had been recrystalized 3 times (BS). One set of MJ was fermented with starters, *Bacillus subtilis* JS2 and *Tetragenococcus halophilus* BS1-37 (each 6 log CFU/g), and another set without starters for 42 days at 10°C. The LAB count of the SSMJ (non-starter) was highest at day 28 (2.30 log CFU/g). The pH of the PSMJ and SSMJ was 5.72–5.77 at day 0, and 5.40–5.50 at day 42. BSMJ showed higher pH and lower titratable acidities than other samples. Amino-type nitrogen (ANN) increased continuously, and SSMJ showed higher values than other samples from day 14. Bacterial species of non-starter MJ were examined by culture independent method. Clone libraries of 16S rRNA genes were constructed in *Escherichia coli* from total DNA from non-starter MJ samples at day 0, 14, and 28. Thirty clones per each sample were randomly selected and DNA sequences were analyzed. *Variovorax* sp., uncultured bacterium, and *Acidovorax* sp. were the most dominant group at day 0, 14, and 28, respectively. *Lactobacillus sakei* and *Streptococcus* sp. were the next dominant group in SSMJ at day 28. A *Streptococcus* sp. was detected from PSMJ at day 28. Sensory evaluation for MJ samples at day 28 showed that SSMJ got higher overall acceptability scores. These results showed that solar salt can cause desirable changes in the microbial community of fermented foods, thereby positively affecting their overall quality.

Keywords: Meongge jeotgal, bacterial community, solar salt, LAB

Introduction

Meongge or common sea squirt (*Halocynthia roretzi*), is a popular seafood in Korea because of its unique flavor and taste. It is commercially grown on a large scale along the southern coastal regions of the Korea peninsula. Cultivated meongge is harvested during the spring season and the fleshy part inside the hard skin is consumed without further processing. Although its unique flavor and taste enable its raw consumption, development of processed products is desirable to increase its year-round sale and commercial value [1, 2]. For this purpose, jeotgal and sikhae (fermented fish) have been prepared from meongge, and the changes in nutrients and taste during fermentation were reported [2, 3]. However, no studies have been done on the microorganisms during meongge jeotgal (MJ) fermentation. In addition, no studies have been done on the effects of salts on MJ fermentation. Salt is an essential ingredient for fermented foods, including jeotgal, where a large amount of salt (20–30%, w/w) is added to prevent putrefaction [4]. Different types of salts such as purified salt, solar salt and bamboo salt are used for fermented foods including kimchi, soybean paste, and jeotgal [5–7]. But the effects of salt types on the quality of fermented foods are not well understood. Especially, the effects of salt types on the growth of microorganisms during

fermentation processes are rarely studied although salt types affect microbial communities, which in turn affect the quality of fermented foods [7, 8]. For the production of high quality fermented foods, the fermentation conditions should be optimized for each type of food, including the concentration and type of salt, use of suitable starters, and temperature. In this work, MJ samples (12% NaCl, w/w) were prepared with 3 different salts; purified salt (PS), 3-year-old solar salt (SS), and bamboo salt (BS, 3rd). MJ samples were fermented for 42 days at 10°C. The growth of bacilli and lactic acid bacteria (LAB) were examined together with other properties during fermentation. Bacterial communities were analyzed by a culture-independent method. The results obtained through this work might be useful as basic data for the preparation of MJ with high quality.

Materials and Methods

Preparation of MJ Samples

Meongge (sea squirt, *Halocynthia roretzi*) was purchased from a local fish market (Korea) in February 2018. Immediately after purchase, the meongge was washed under running tap water, and left standing for 10 min to remove excess water. Each 2.0 kg of meongge was mixed with salt. NaCl concentration was adjusted to 12% (w/w) by adding different amounts of each salt: 275.86 g for purified salt (PS, Hanju, Korea, 2017, NaCl 99%); 324.19 g for solar salt aged for 3 years (SS, Taepyung salt farm, Sinan, Korea, NaCl 86.03%); 290.77 g for bamboo salt (BS, Insanga, Korea, melted and recrystallized 3 times, NaCl 94.54%). For one set of MJ samples, *Bacillus subtilis* JS2 and *Tetragencoccus halophilus* BS1-37 were inoculated at each 6 log CFU/g (starter MJ). Another set of MJ samples were prepared without starters (non-starter MJ). All 6 MJ samples were fermented for 42 days at 10°C and analyzed every 7 days during fermentation.

Viable Cell Counting

Twenty gram of MJ sample was mixed with 20 ml of peptone water (0.1%, w/v) and homogenized using a Stomacher 80 (Seward, USA). Homogenate was filtered with a bag filter (3M, 19 × 30, USA) and diluted serially with peptone water. Diluted samples were spread on lactobacilli MRS agar (Difco, USA) plates for lactic acid bacteria (LAB) counting, Luria-Bertani (LB) agar (BD Biosciences, USA) plates for bacilli counting, and yeast-mold (YM, BD Biosciences) agar plates for yeast counting. Plates were incubated for 72 h at 37°C for bacilli, and 120 h at 30°C for LAB and yeasts.

pH and Titratable Acidity (TA)

Ten grams of homogenized MJ sample was mixed with 40 ml of distilled water, and shaken for 1 h in a water bath (150 rpm, 30°C). Supernatant was obtained after centrifugation (4,000 × g, 20 min, 4°C). The pH of the supernatant was measured using a pH meter (DP-215M, DMS, Korea). Titratable acidity (TA) was calculated by titrating supernatant with 0.1 N NaOH until pH 8.4. The amount of NaOH was used to calculate the amount of lactic acid (%).

Amino-Type Nitrogen (ANN), Ammonia-Type Nitrogen (AMN) and Volatile Basic Nitrogen (VBN)

ANN, AMN, and VBN of MJ samples were measured during fermentation according to methods described previously [7].

Moisture Content and Salinity

Moisture content of homogenized MJ sample was measured by using an infrared moisture analyzer (MX-50, AND, Japan). For salinity measurements, 10 g of homogenized MJ sample was mixed with 40 ml of distilled water. Supernatant was obtained after shaking in a water bath and centrifugation as stated above. Salinity of supernatant was measured by using a salt-meter (PAL-SALT, Atago, Japan). Measurements were repeated 3 times and the average values were shown.

Bacterial Communities of Non-Starter MJ Samples

Non-starter MJ samples were collected at day 0, 14, and 28. Total DNA was extracted by using a EZ-10 Spin Column Soil DNA Mini-prep Kit (Bio Basic Inc., Canada). 16S rRNA genes were amplified from the extracted DNA samples by using universal primer pair, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGY TACCTTACGACTT-3′). PCR was done under the following conditions: denaturation at 94°C for 5 min, 40 cycles of 30 sec at 94°C, 2 min at 57°C, and 2 min at 72°C, and a final extension at 72°C for 7 min. Amplified fragments were purified by using a FavorPrep PCR Purification Kit (Favorgen, Taiwan) and ligated with pGEM-T easy vector (Promega, USA). *Escherichia coli* DH5α competent cells (Enzynomics, Korea) were transformed with the ligation mixture, and transformants were selected on LB plates with ampicillin (100 μg/ml), isopropyl β-D-1-thiogalactopyranoside (500 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg/ml). Thirty colonies were selected randomly from each MJ sample, and their inserts were sequenced. DNA sequencing was done at Cosmogenetech (Korea), and BLAST was used to find homologous sequences in the data library (http://www.ncbi.nlm.nih.gov/BLAST).

Sensory Evaluation

Sensory evaluation for MJ samples at day 28 was done by 21 tasters (male:female = 8:13, average age, 25.7 years) using a 5-point scale for color, flavor, taste, texture, saltiness, sourness, bitterness, fishy smell, umami and overall acceptability. The properties were evaluated as follows: very poor (1 point), medium (3 points), and very good (5 points). Tasters were provided with drinking water and crackers to rinse their mouths and remove any aftertaste before testing other samples. The statistical analyses of sensory data were performed by least significant difference (LSD) test ($p < 0.005$).

Results and Discussion

Viable Cell Counting

Viable cells of bacilli, LAB and yeasts were counted every 7 days during 42 days of fermentation and the results are shown in Fig. 1 and Fig. 2. Yeasts were not detected during the entire fermentation period. Bacilli and LAB counts of starter MJ samples decreased gradually from day 0 (immediately after preparation) until day 42. The initial bacilli counts were 6.56–6.87 log CFU/g and the final counts were 5.61–5.83 log CFU/g, one log reduction during fermentation. LAB counts decreased in a similar way but decreased more, the initial counts of 6.19–6.40 log CFU/g decreased to 4.71–4.79 log CFU/g at day 42 (Fig. 1). Considering the inoculum size of *B. subtilis* JS2 and *T. halophilus* BS1-37 (each 6 log CFU/g), the results indicated that added starters did not grow well under the fermentation conditions (10°C and 12% NaCl (w/w)) although significant portions of starter cells survived. In previous studies, *B. subtilis* JS2 grew up to 20% NaCl (w/v) in LB broth [9] and *T. halophilus* BS1-37 grew up to 23% NaCl (w/v) in lactobacilli MRS broth [10]. MJ fermentation conditions used for this work, especially low temperature (10°C) combined with relatively high salt concentration, were not good for the growth of starters. MJ samples prepared with different salt types did not show significant differences in bacilli and LAB viable counts.

Bacilli counts of non-starter MJ samples were 4.07–5.15 log CFU/g at day 0, and decreased during fermentation (Fig. 2). Bacilli counts of PSMJ and SSMJ samples decreased rapidly during the first week and the counts were 2.77–2.82 log CFU/g, the lowest counts, at day 7. BSMJ showed the lowest count (2.74 log CFU/g) at day 21. After the lowest points, bacilli counts increased slowly and reached to 4.20–4.64 log CFU/g at day 42. The final counts were either slightly higher than initial counts for PSMJ and BSMJ

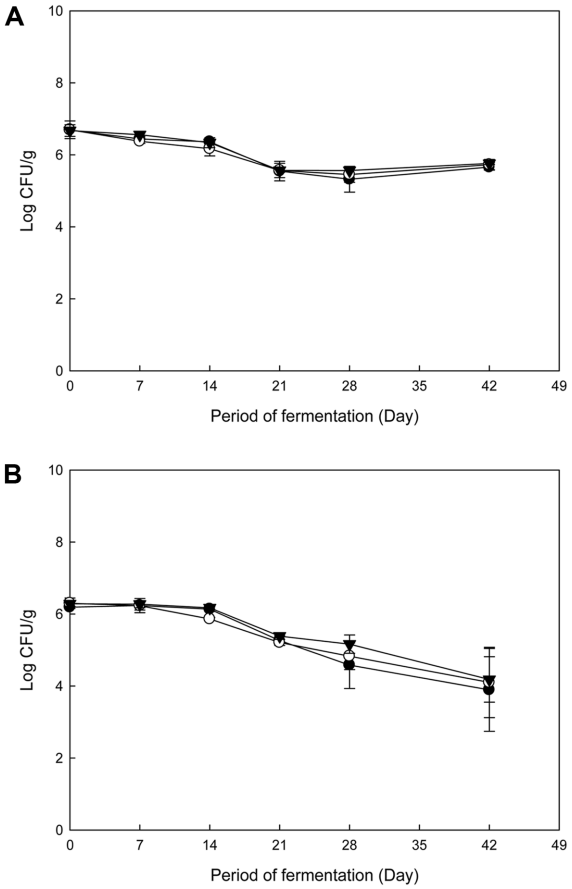

Fig. 1. Bacilli (**A**) and LAB (**B**) viable counts of starter MJ samples during fermentation.

●, starter MJ with purified salt; ○, starter MJ with solar salt; ▼, starter MJ with bamboo salt.

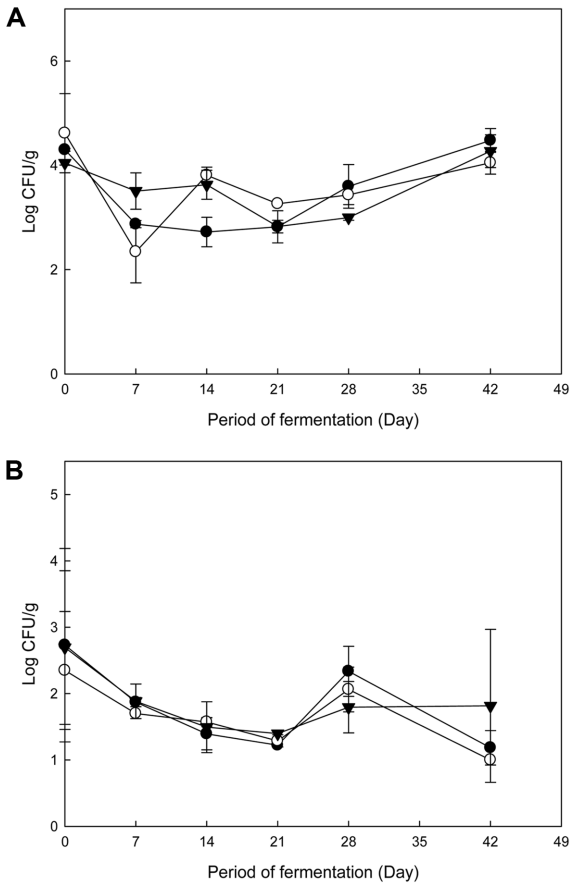

Fig. 2. Bacilli (**A**) and LAB (**B**) viable counts of non-starter MJ samples during fermentation.

● , non-starter MJ with purified salt; ○ , non-starter MJ with solar salt; ▼, non-starter MJ with bamboo salt.

samples or 1 log reduced for SSMJ. LAB counts of non-starter MJ samples were 2.98–3.76 log CFU/g at day 0 and gradually decreased during fermentation. The final LAB counts were 1 log CFU/g. The final LAB counts of non-starter MJ samples were 5,000 times on average lower than those of starter MJ samples, whereas the final bacilli counts of non-starter MJ samples were 10 times lower than those of starter MJ samples. No significant differences in bacilli and LAB counts were observed among non-starter MJ samples.

pH and Titratable Acidity (TA)

pH and TA values of MJ samples during fermentation are shown in Fig. 3. Immediately after preparation, pH of PSMJ and SSMJ samples were 5.72–5.77, and pH decreased slowly until day 14 (5.53–5.56), then increased rapidly until day 21 (5.82–5.87) (Fig. 3A). After day 21, pH again decreased gradually, reaching to 5.40–5.50 at day 42. The final pH values were lower than those of the initial values by 0.2–0.3. BSMJ had much higher pH values of 8.40 ± 0.02 (starter) and 8.29 ± 0.01 (non-starter) at day 0. This was due to the high mineral content of BS. BS contains a large amount of K, Si, Fe, and PO_4 and its aqueous solution is alkaline (pH 10) [11]. Unlike other MJ samples, pH of the BSMJ samples decreased continuously throughout the fermentation period, and the final pH was 7.29 for starter and 7.01 for non-starter BSMJ. BSMJ (starter and non-starter) showed higher pH values throughout the fermentation period. At day 42, pH of PSMJ was 5.50 for starter and 5.47 for non-starter. pH of SSMJ was 5.40 for starter and 5.43 for non-starter. Starter SSMJ showed the lowest pH value.

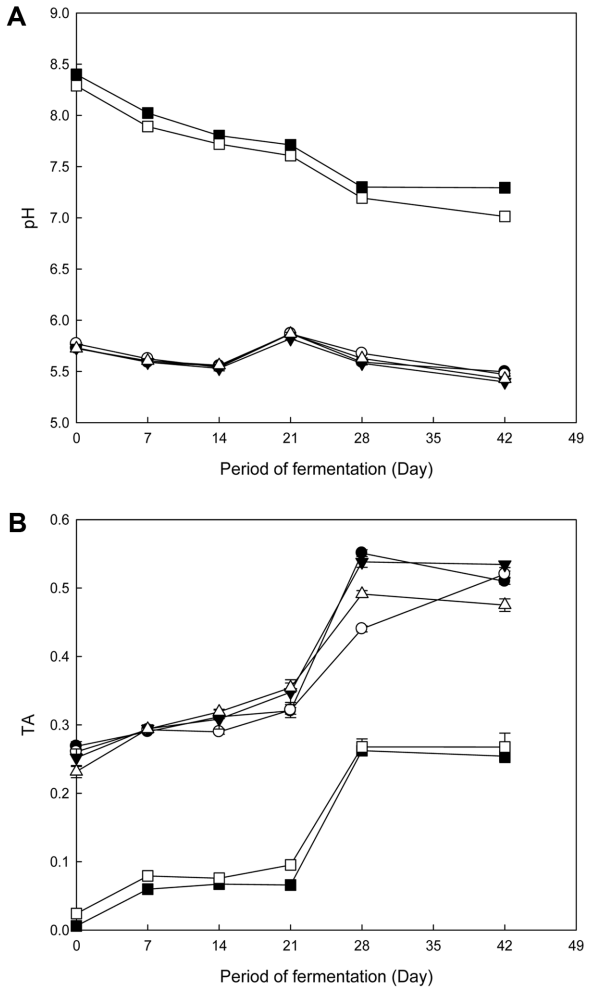

Fig. 3. pH (**A**) and titratable acidity (**B**) of MJ samples during fermentation.

●, starter MJ with purified salt; ○, non-starter MJ with purified salt; ▼, starter MJ with solar salt; △, non-starter MJ with solar salt; ■, starter MJ with bamboo salt; □, non-starter MJ with bamboo salt.

TA values of PSMJ and SSMJ samples were 0.23–0.27, but just 0.01–0.02 for BSMJ at day 0 (Fig. 3B). TA values of all MJ samples increased gradually until day 21, and then rapidly increased until day 28. After day 28, TA values either increased or decreased slightly. At day 42, TA was 0.25 for starter and 0.27 for non-starter BSMJ. TA of PSMJ was 0.51 for starter and 0.52 for non-starter, and TA of SSMJ was 0.53 for starter and 0.48 for non-starter. Starter PSMJ and SSMJ showed the highest TA values. pH and TA values of jeotgal samples reflect the amounts of organic acids such as lactic acid produced by microorganisms during fermentation [12, 13].

ANN, AMN, and VBN Content

Amino-type nitrogen (ANN) of MJ samples were measured during fermentation (Fig. 4A). ANN is related to the degree of protein hydrolysis of raw materials [14]. Proteins in the raw materials are hydrolyzed by proteolytic enzymes either from microorganisms or raw materials into peptides and amino acids, causing the development of unique physical properties, flavor, and aroma of fermented foods [15]. The ANN was 118.53–145.47 mg% at day 0, immediately after preparation. ANN of all MJ samples increased rapidly during fermentation. As expected, starter MJ showed higher values than non-starter MJ. The starters (*Bacillus subtilis* JS2 and *Tetragenococcus halophilus* BS1-37) possess strong proteolytic activities. Starter SSMJ showed the highest values from day 14 until day 42, 257.12 ± 4.19 mg% at day 42. BSMJ showed the lowest values until day 28 (203.14–209.86 mg% at day 28). But after day 28, the ANN values increased rapidly, and were same as in other MJ samples at day 42.

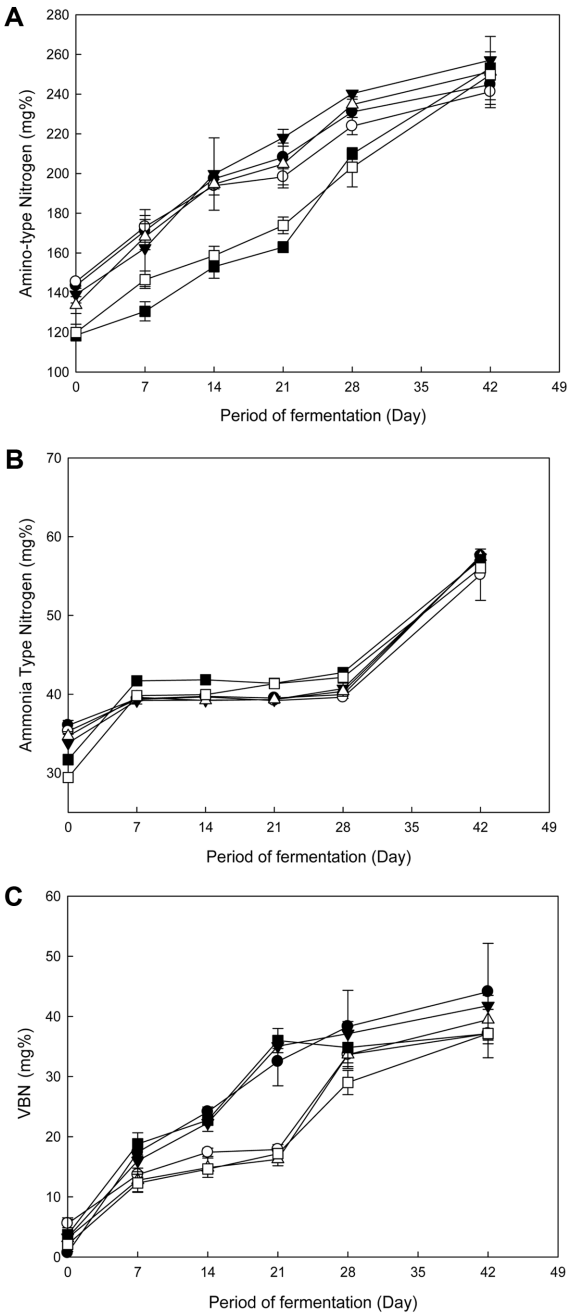

Fig. 4. Amino-type nitrogen (**A**), ammonia-type nitrogen (**B**), and volatile basic nitrogen (**C**) of MJ samples during fermentation.

●, starter MJ with purified salt; ○, non-starter MJ with purified salt; ▼, starter MJ with solar salt; △, non-starter MJ with solar salt; ■, starter MJ with bamboo salt; □, non-starter MJ with bamboo salt.

The delicate flavors of fermented foods are developed as proteins are hydrolyzed into peptides and amino acids. However, if excessive decomposition occurs, strongly volatile and unpleasant ammonia-type nitrogens are generated, causing a negative effect. But ammonia-type nitrogen also serves as a fermentation index of fermented foods. The AMN levels of MJ during fermentation are shown in Fig. 4B. The AMN levels of starter MJ were 31.49–57.83 mg%, and those of non-starter MJ were 29.28–57.94 mg% at day 0. AMN increased rapidly until day 7, then did not change until day 28, and then increased rapidly again until day 42. BSMJ showed higher AMN than other MJ samples until day 28. The AMN levels of starter MJ were not different significantly from those of non-starter MJ.

Volatile basic nitrogen (VBN) levels of MJ were measured (Fig. 4C). VBN represents lower basic nitrogen compounds with volatility, such as various basic amines including ammonia and trimethylamine, and the value is used for assessing the freshness of foods [16]. VBN levels of MJ were 0.70–6.27 mg% at day 0. All MJ samples showed steady increases from day 0 until day 42. Especially, VBN levels of starter MJ samples increased rapidly compared to those of non-starter MJ samples. PSMJ showed the highest value at day 28 (starter 38.31 ± 6.03, non-starter 33.67 ± 2.01). BSMJ showed the lowest value from day 28 until the end of fermentation.

Moisture Content and Salinity

The moisture content of MJ samples at day 0 was 73.99–76.35% (Fig. 5A). Raw meongge contains more than 80% moisture (https://www.nifs.go.kr/page?id=aq_seafood_2_7&type=tot&from=totList&fim_col_id=2009-MF0008526-6-D01). The result showed that some water loss occurred due to added salt. In a previous report, the moisture content of MJ with 8% NaCl was 75.10 ± 0.2% [17]. Our results are quite similar with that report.

The initial salinities of MJ samples were not constant but became constant as fermentation proceeded (Fig. 5B). Salinities of BSMJ (non-starter) were higher than other samples until day 21 (12.53–13.43%). Salinities of MJ samples were fluctuated until day 21 (11.35–13.43%) and then became stabilized from day 28 (11.98–12.70%). At day 42, non-starter and starter BSMJ showed higher salinities than other MJ, and the value was 12.40 ± 0.36% for non-starter and 12.33 ± 0.55% for starter BSMJ.

Bacterial Communities of Non-Starter MJ Samples

Thirty clones of each clone library were sequenced and about 800 nucleotide sequences were analyzed by BLAST for identification. *Variovorax* sp. was the most dominant group in non-starter MJ samples at day 0, occupying 30%,

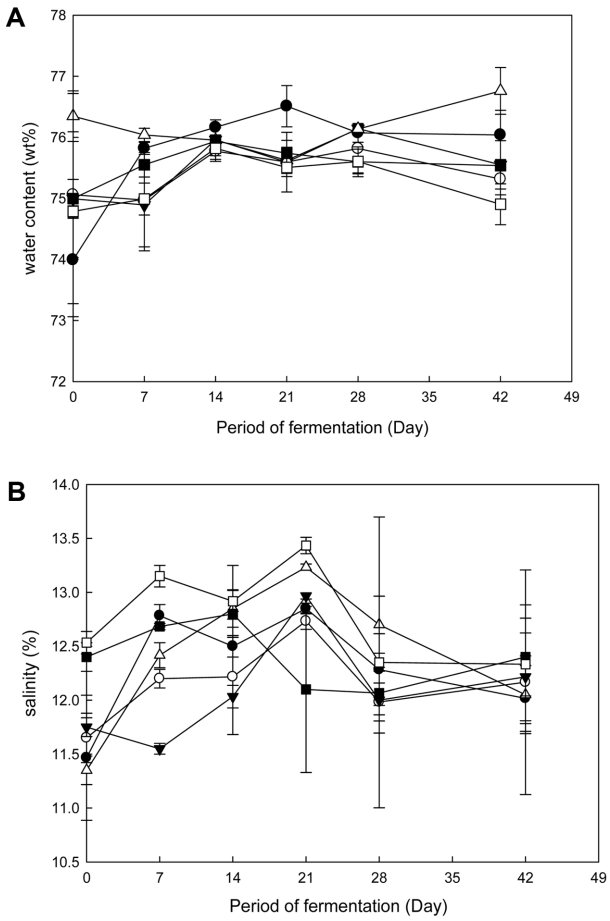

Fig. 5. Moisture content (**A**) and salinity (**B**) of MJ samples during fermentation.
●, starter MJ with purified salt; ○, non-starter MJ with purified salt; ▼, starter MJ with solar salt; △, non-starter MJ with solar salt; ■, starter MJ with bamboo salt; □, non-starter MJ with bamboo salt.

46.7% and 40% of PSMJ (9 out of 30), SSMJ (14 out of 30) and BSMJ (12 out of 30) clone library, respectively (Table 1). *Variovorax* is a genus belonging to the family of Comamonadaceae, gram negative, motile, and widely present in various environments of soil and water including ocean sediment near the Arctic [18, 19]. They possess diverse metabolic capacities and are considered promising organisms for bioremediation [20]. *Variovorax* sp. were likely to be originated from sea squirt since day 0 was just after preparation of MJ samples. Since some *Variovorax* sp. are known to tolerate NaCl up to 3–5%, and the NaCl concentration of MJ samples was 12%, *Variovorax* sp. were unlikely to grow in MJ samples [21]. This seemed the reason of reduced detection of *Variovorax* sp. at day 14 and no detection at day 28 (Tables 2 and 3). *Acidovorax* sp.

Table 1. Identification of clone library prepared from non-starter MJ (0 day).

Sample	16S rRNA gene sequence* (NCBI Accession No.)	Similarity (%)	Clone numbers out of 30 (%)
PS	*Variovorax* sp. (MH698893.1)	99%	8 (26.7%)
	Variovorax sp. (LC040880.1)	99%	1 (3.3%)
	Acidovorax sp. (MH512862.1)	99%	6 (20.0%)
	Burkholderia cepacia (LC420066.1)	99%	4 (13.3%)
	Bradyrhizobiaceae bacterium (AY429694.1)	98%	2 (6.7%)
	Burkholderia sp. (CP029825.1)	100%	1 (3.3%)
	Burkholderia cepacia (LC420066.1)	99%	1 (3.3%)
	Rhodopseudomonas sp. (MG798777.1)	93%	1 (3.3%)
	Methylobacterium sp. (MH686068.1)	99%	1 (3.3%)
	Bradyrhizobium sp. (MG798777.1)	99%	1 (3.3%)
	Ralstonia sp. (LC385700.1)	99%	1 (3.3%)
	Ralstonia pickettii (CP001645.1)	99%	1 (3.3%)
	Uncultured bacterium (MH252192.1)	99%	1 (3.3%)
	Uncultured bacterium (HM141889.1)	99%	1 (3.3%)
Total	30		
SS	*Variovorax* sp. (MH698893.1)	99%	7 (23.3%)
	Variovorax sp. (EU057880.1)	98%	2 (6.7%)
	Variovorax sp. (GQ478274.1)	99%	1 (3.3%)
	Variovorax sp. (LC040880.1)	99%	1 (3.3%)
	Variovorax boronicumulans (MH929813.1)	99%	1 (3.3%)
	Variovorax boronicumulans (MH211313.1)	100%	1 (3.3%)
	Variovorax paradoxus (GU186109.1)	99%	1 (3.3%)
	Acidovorax sp. (MH512862.1)	100%	4 (13.3%)
	Burkholderia cepacia (LC420066.1)	99%	6 (20.0%
	Comamonas sp. (MH698867.1)	100%	1 (3.3%)
	Comamonas sp. (MG561176.1)	98%	1 (3.3%)
	Burkholderia lata (CP013406.1)	100%	2 (6.7%)
	Burkholderia cenocepacia (KF475841.1)	100%	1 (3.3%)
	Uncultured bacterium (JX225058.1)	98%	1 (3.3%)
Total	30		
BS	*Variovorax* sp. (MH698893.1)	100%	11 (36.7%)
	Variovorax sp. (LC040880.1)	100%	1 (3.3%)
	Acidovorax sp. (MH512862.1)	100%	5 (16.7%)
	Acidovorax sp. (KT354276.1)	99%	1 (3.3%)
	Uncultured bacterium (JN820216.1)	99%	1 (3.3%)
	Uncultured bacterium (KF515167.1)	99%	2 (6.7%)
	Uncultured bacterium (KR110096.1)	99%	2 (6.7%)
	Uncultured bacterium (HM141889.1)	99%	1 (3.3%)
	Burkholderia lata (CP013406.1)	100%	2 (6.7%)
	Bradyrhizobium sp. (AB681389.1)	99%	1 (3.3%)
	Bradyrhizobium elkanii (FN178446.1)	94%	1 (3.3%)
	Afipia sp. (AB586143.1)	99%	1 (3.3%)
	Pseudomonas sp. (MH428812.1)	99%	1 (3.3%)
Total	30		

*Approximately 800 nucleotides were read.

Table 2. Identification of clone library prepared from non-starter MJ (14 days).

Sample	16S rRNA gene sequence* (NCBI Accession No.)	Similarity (%)	Clone numbers out of 30 (%)
PS	*Variovorax* sp. (MH698893.1)	99%	8 (26.7%)
	Uncultured bacterium (HM141889.1)	99%	6 (20.0%)
	Uncultured bacterium (FN567167.1)	96%	1 (3.3%)
	Uncultured bacterium (DQ451505.1)	99%	1 (3.3%)
	Uncultured bacterium (JX564435.1)	94%	1 (3.3%)
	Sphingopyxis sp. (KM670029.2)	99%	1 (3.3%)
	Sphingopyxis sp. (KX085480.1)	99%	1 (3.3%)
	Serratia marcescens (CP029715.1)	99%	1 (3.3%)
	Serratia marcescens (CP027798.1)	99%	1 (3.3%)
	Ralstonia sp. (HE575954.1)	96%	1 (3.3%)
	Ralstonia sp. (LC385700.1)	100%	1 (3.3%)
	Bradyrhizobium sp. (MG798777.1)	98%	1 (3.3%)
	Byrhizobium sp. (MG798777.1)	92%	1 (3.3%)
	Burkholderia lata (CP013406.1)	100%	1 (3.3%)
	Comamonas sp. (MH698867.1)	99%	1 (3.3%)
	Afipia sp. (JX963091.1)	99%	1 (3.3%)
	Acinetobacter sp. (FN395271.1)	99%	1 (3.3%)
	Sphingomonas sanxanigenens (AB649022.1)	100%	1 (3.3%)
Total	30		
SS	Uncultured bacterium (HM141889.1)	99%	19 (63.3%)
	Uncultured bacterium (GU455124.1)	99%	1 (3.3%)
	Afipia sp. (EU130950.1)	99%	3 (10.0%)
	Afipia sp. (JN697519.1)	99%	2 (6.7%)
	Afipia sp. (KU713085.1)	99%	1 (3.3%)
	Afipia lausannensis (DQ123622.1)	99%	1 (3.3%)
	Afipia massiliensis (NR_025646.1)	100%	1 (3.3%)
	Uncultured Afipia sp. (JN697519.1)	100%	1 (3.3%)
	Sphingomonas sanxanigenens (AB649022.1)	99%	1 (3.3%)
Total	30		
BS	Uncultured bacterium (HM141889.1)	99%	10 (33.3%)
	Uncultured bacterium (JF429287.1)	99%	1 (3.3%)
	Uncultured bacterium (DQ890427.1)	97%	1 (3.3%)
	Variovorax sp. (MH698893.1)	100%	2 (6.7%)
	Afipia sp. (JX963091.1)	99%	1 (3.3%)
	Afipia sp. (EU130950.1)	99%	1 (3.3%)
	Afipia massiliensis (KY319044.1)	99%	1 (3.3%)
	Bradyrhizobium sp. (CP025113.1)	94%	2 (6.7%)
	Bradyrhizobium sp. (MG798777.1)	99%	2 (6.7%)
	Hyphomonas sp. (CP016437.1)	94%	3 (10.0%)
	Sphingopyxis macrogoltabida (CP012700.1)	89%	2 (6.7%)
	Serratia sp. (KF077052.1)	94%	1 (3.3%)
	Comamonas testosteroni (LC090503.1)	98%	1 (3.3%)
	Pseudomonas mendocina (CP010892.1)	97%	1 (3.3%)
	Rhodopseudomonas palustris (CP000463.1)	91%	1 (3.3%)
Total	30		

*Approximately 800 nucleotides were read.

and *Burkholderia* sp. were the next abundant groups in PSMJ, both occupying 20% of a clone library (6 out of 30). *Acidovorax* sp. were also detected often from SSMJ (13.3%) and BSMJ (20%) clone libraries. The genus *Acidovorax* consists of Gram-negative bacteria belonging to the family of Comamonadaceae, the order of Burkholderiales, and of β-proteobacteria. *Acidovorax* sp. can be separated into 2 groups, phytopathogens and water and soil inhabitants [22]. Some *Acidovorax* sp. have been studied for their ability to degrade chemical pollutants such as arsenite, poly-chlorinated biphenyl, and 2-nitrotoluene [23, 24]. *Burkholderia* sp. were also detected often, occupying 20% (6 out of 20), 30% (9 out of 30), and 6.7% (2 out of 30) of PSMJ, SSMJ, and BSMJ clone libraries, respectively. *Burkholderia* sp. are widely distributed in various environments including soil, water, wastewater treatment systems, and plants [25]. Uncultured bacteria occupied 6.7% (2 out of 30), 3.3% (1 out of 30), and 20% (6 out of 30) of PSMJ, SSMJ, and BSMJ clone libraries, respectively. *Ralstonia* sp. and *Bradyrhizobiaceae* sp. were detected twice (6.7%) in the PSMJ clone library. *Comanmonas* sp. were detected twice (6.7%) from the SSMJ clone library, and a *Pseudomonas* sp. was detected once (3.3%) from the BSMJ clone library.

From MJ samples at day 14, uncultured bacteria were the major group, occupying 30%, 66.7%, and 40% of the PSMJ, SSMJ, and BSMJ clone libraries, respectively (Table 2). In the PSMJ clone library, *Variovorax* sp. were the next dominant (8 out of 30, 26.7%), and *Serratia* sp., *Ralstonia* sp., and *Bradyrhizobium* sp. were detected twice (6.7%). In the SSMJ clone library, *Afipia* sp. were the second dominant (8 out of 30, 26.7%). *Afipia* is a genus Gram-negative and nonfermentative rod-shaped bacteria belonging to the family Bradyrhizobiaceae, α-proteobacteria. *Afipia* sp. are studied for their ability to degrade toxic chemicals such as 1,4-dioxane [26]. In the BSMJ clone library, *Bradyrhizoboum* sp. were the second dominant (4 out of 30, 13.3%), and *Afipia* sp. and *Hyphomonas* sp. were detected 3 times (10%).

From MJ samples at day 28, *Acidovorax* sp. were the most dominant, occupying 80%, 40%, and 73.3% of PSMJ, SSMJ, and BSMJ clone libraries, respectively (Table 3). Uncultured bacteria were the second most dominant. *Burkholderia* sp. were detected 3 times (10%) from BSMJ clone library, and once each from PSMJ and SSMJ clone libraries. *Afipia* sp. were observed from MJ samples at day 0 and 14, but not at 28.

LAB, *Lactobacillus sakei* and *Streptococcus* sp., were detected from PSMJ and SSMJ clone libraries at day 28. LAB occupied 30% (*L. sakei* 16.7%, *Streptococcus* sp. 13.3%) of the SSMJ clone library and 3.3% of the PSMJ clone library, but were not detected from BSMJ. It seemed that environments

Table 3. Identification of clone library prepared from non-starter MJ (28 days).

Sample	16S rRNA gene sequence (NCBI Accession No.)	Similarity (%)	Clone numbers out of 30 (%)
PS	*Acidovorax* sp. (MH512862.1)	100%	22 (73.3%)
	Acidovorax sp. (AY093698.1)	100%	1 (3.3%)
	Acidovorax sp. (KT354276.1)	100%	1 (3.3%)
	Bacterium enrichment (KC979079.1)	98%	2 (6.7%)
	Ralstonia sp. (MH144298.1)	98%	1 (3.3%)
	Ralstonia sp. (LC385700.1)	99%	1 (3.3%)
	Burkholderia lata (CP013406.1)	100%	1 (3.3%)
	Streptococcus parasanguis (AM157421.1)	100%	1 (3.3%)
Total	30		
SS	*Acidovorax* sp. (MH512862.1)	100%	12 (40.0%)
	Uncultured bacterium (KM852123.1)	99%	5 (16.7%)
	Uncultured bacterium (HM141889.1)	100%	2 (6.7%)
	Lactobacillus sakei (CP032652.1)	99%	4 (13.3%)
	Lactobacillus sakei (AB362606.1)	99%	1 (3.3%)
	Streptococcus pyogenes (LS483331.1)	100%	2 (6.7%)
	Streptococcus pyogenes (CP022206.1)	100%	2 (6.7%)
	Burkholderia cepacia (LC420066.1)	99%	1 (3.3%)
	Piscinibacter sp. (KU233249.1)	100%	1 (3.3%)
Total	30		
BS	*Acidovorax* sp. (MH512862.1)	100%	22 (73.3%)
	Uncultured bacterium (KF515167.1)	99%	2 (6.7%)
	Uncultured bacterium (KM852123.1)	99%	1 (3.3%)
	Uncultured bacterium (KU973397.1)	99%	1 (3.3%)
	Uncultured bacterium (KR110096.1)	100%	1 (3.3%)
	Burkholderia cepacia (LC420066.1)	99%	2 (6.7%)
	Burkholderia lata (CP013406.1)	99%	1 (3.3%)
Total	30		

*Approximately 800 nucleotides were read.

favoring the growth of LAB were developed in SSMJ around day 28, and solar salt might encourage the growth of LAB. The appearance of LAB might cause changes in pH and TA of MJ samples. pH values decreased at day 21 and TA values increased rapidly from day 21 until day 28 (Fig. 3). In a previous report, bacterial communities of commercial jeotgal varieties made with myeolchi (anchovy), meongge (common sea squirt), and saeu (small shrimp) were analyzed by pyrosequencing of the V1-V2 region of 16S rRNA genes [27]. *L. sakei* was the predominant bacterial species (50.7%, average relative abundance) of 5 types of meongge jeotgal purchased at 4 different cities on the east and south coasts in Korea. *Lactobacillus curvatus* (15.8%) and *Weissella koreensis* (8%) were the next dominant species. The results matched with our observation of SSMJ at day 28. LAB, especially *L. sakei*, seemed an important member of the bacterial community in MJ fermentation. But it should be mentioned that the 5 commercial meongge jeotgal samples were quite different from ours. The average NaCl concentration was 4.6%, much lower than our samples (12%). Also, the average pH was 4.4 which was also lower than those of ours. The pH of PSMJ and SSMJ was 5.4–5.5 at day 42 (Fig. 3). Commercial meongge jeotgal products used for the previous study were most likely fermented for just a few days considering their low salt contents. The procedures of fermentation were also unknown. Because of high salinity and low temperature (10°C), LAB grew slowly in our MJ samples, and this explained the low LAB counts and higher pH values of our samples. At day 0, LAB counts of our non-starter MJ samples were 9.50×10^2–5.75×10^3 CFU/g, and the numbers decreased to 1×10^1 CFU/g at day 42. But LAB counts of 5 commercial samples were 3.9×10^6–6.5×10^7 CFU/g [27]. Unlike previous studies, *Tetragenococcus* sp. were not detected from our MJ samples up to day 28. In a previous study, fish sauce was prepared from deep sea smelt (15% NaCl, w/w) and fermented at room temperature for 8 months [13]. When bacterial communities were analyzed by clone library (the same method used by us), *Staphylococcus* sp. were the dominant group until 4 weeks, and then *T. halophilus* became the most dominant species [13]. In our study, *Staphylococcus* sp. and *Tetragenococcus* sp. were not detected up to day 28. It was possible that *Tetragenococcus* sp. might appear in MJ samples after day 28 considering the low temperature (10°C). In another study with commercial galchi jeotgal and myeolchi jeotgal, *T. halophilus*, *T. muriaticus*, and *Lactobacillus sakei* were the predominant species determined by PCR-DGGE method [28]. Salinities of these jeotgal types were very high, 30-48% for galchi jeotgal and 26-40% for myeolchi jeotgal. Again, the exact fermentation conditions were unknown for these commercial products. In another study, kimchi samples were prepared with different jeotgal and their LAB were compared by culture independent method [29]. *L. sakei* was the dominant LAB in a kimchi with saeu jeotgal and *T. halophilus* was the dominant in kimchi with myeolchi jeotgal. The results indicate that growth of LAB in kimchi is affected by the particular jeotgal type added into kimchi.

One question to be answered was that bacilli were not detected from our MJ samples by culture-independent

method. Bacilli counts of non-starter MJ samples were 5.52×10^2–1.42×10^5 CFU/g throughout fermentation. It was suspected that universal primers (27F and 1492R) used were not efficient for amplification of 16S rRNA genes from bacilli. Kim *et al.* reported that bacilli were not detected from doenjang samples by PCR-DGGE where 16S rRNA genes were first amplified by universal primers (27F and 1492R) followed by a second nested PCR using internal primers (338F and 518R) [30]. Bands specific for bacilli were only detected when a second nested PCR was done by using bacilli specific primers [30]. Fukui *et al.* also reported similar results [13]. Bacilli were not detected from fish sauce prepared from deep sea smelt by 16S rRNA gene clone library method where 27F and 1492R primers were used, but detected by cultural methods. These results showed that 16S rRNA genes of bacilli were poorly amplified with universal primers (27F and 1492R), which was confirmed by our results. Therefore, care must be exercised when community analysis is planned by culture-independent method.

We used cultural method to isolate bacilli from MJ samples which were taken out at day 28 and frozen at −70°C. MJ samples were thawed and diluted with 0.1% peptone water and then spreaded onto marine agar (Difco, USA, 2.5% NaCl) and MRS agar plates. Colonies were picked up and 16S rRNA genes were amplified by using 27F and 1492R universal primers. Sequencing results showed that bacilli were the most dominant group, occupying 50% (15 out of 30), 66.7% (20 out of 30), and 80% (24 out of 30) of 30 sequenced colonies on marine agar from PSMJ, SSMJ and BSMJ, respectively (Table S1). Not many colonies appeared on MRS agar plates. *Enterococcus faecium* and *Staphyococcus epidermidis* were identified together with bacilli (Table S2). The MJ samples were stored at −70°C, and this may have caused significant death of bacteria. Our results clearly show that culture-independent method should be used together with cultural method for accurate community analysis.

In previous studies on bacterial communities of commercial jeotgal, communities at a certain stage of fermentation were examined. No information on the changes in bacterial communities can be obtained from such purchased products. In this respect, our work provided for the first time some useful hints on the changes in bacterial communities of MJ samples during fermentation under a specific condition (12% salt and 10°C). It became clear that bacterial community is affected by many factors including the nature of main materials, salt concentration, salt type, temperature, and other environmental factors.

The 2 dominant groups, *Acidovorax* sp. and LAB, were likely to play some roles in the fermentation of MJ, especially in SSMJ around day 28. Although no studies on the roles of *Acidovorax* sp. (or any other group) for jeotgal fermentations have been reported, results obtained through this work are interesting and show the need for future studies. Although just 30 clones from each MJ sample were examined, the results still showed significant differences in bacterial species among MJ samples prepared with different types of salts. Since each salt type has different components, and bacteria such as LAB respond to specific components, difference in bacterial community (and quality of fermented foods) can be expected [31]. Future studies on the roles of *Acidovorax* sp. and other groups identified through this work are necessary. Studies should include isolation of these organisms by cultural methods, characterization of properties of these groups, and identification of major metabolites produced by them. Also, more studies are required to confirm the effects of solar salt on the growth of LAB during MJ fermentation.

Table 4. Sensory evaluation results of meongge jeotgal samples at day 28.

After 28 days	Starter			Non-starter		
	PS	SS	BS	PS	SS	BS
Color	4.19 ± 0.87^{c}	3.67 ± 0.66^{c}	2.86 ± 0.96^{b}	3.81 ± 1.08^{c}	3.76 ± 1.04^{c}	2.24 ± 1.14^{a}
Flavor	3.19 ± 0.81^{ab}	3.57 ± 1.12^{b}	2.86 ± 1.28^{ab}	3.00 ± 1.05^{ab}	3.33 ± 1.02^{b}	2.57 ± 0.98^{a}
Texture	3.86 ± 0.79^{bc}	4.10 ± 0.70^{cd}	2.76 ± 0.83^{a}	3.71 ± 0.85^{bc}	3.38 ± 0.86^{b}	3.33 ± 0.73^{b}
Saltiness	2.86 ± 1.28^{bc}	3.33 ± 0.86^{c}	2.43 ± 1.36^{ab}	2.33 ± 1.20^{ab}	3.00 ± 1.05^{bc}	2.00 ± 1.22^{a}
Fishy smell	3.10 ± 1.30^{b}	2.81 ± 0.87^{ab}	2.38 ± 1.02^{a}	2.90 ± 0.94^{ab}	3.19 ± 0.81^{b}	2.33 ± 1.02^{a}
Umami	3.24 ± 0.70^{bc}	3.43 ± 1.08^{bc}	2.81 ± 0.81^{ab}	3.48 ± 1.12^{c}	3.43 ± 1.03^{bc}	2.57 ± 0.93^{a}
Overall acceptability	3.29 ± 0.72^{b}	3.48 ± 1.21^{b}	2.48 ± 1.08^{a}	3.19 ± 0.93^{b}	3.43 ± 0.68^{b}	2.48 ± 1.03^{a}

*Significant difference between the values in the same tested items ($p < 0.005$, by least significant difference test).

Sensory Evaluation

Sensory evaluation was done for MJ samples at day 28 (Table 4). SSMJ (non-starter and starter) received the highest scores for flavor, and the overall acceptability scores were also higher than those of other MJ samples. BSMJ samples got lower scores than other types of MJ. This might be related to differences in bacterial communities among MJ samples. For example, LAB were not detected from BSMJ. LAB are the most important group of microorganisms for various fermented foods by producing acids, amino acids, peptides, and other metabolites positively affecting the quality of fermented foods [32]. In this respect, solar salt has an advantage over PS and BS for MJ fermentation by encouraging growth of LAB. These effects might be the major reason for the better sensory properties of SSMJ samples. Further studies are necessary to characterize the responsible compounds present in SS. If the compounds are identified, they could be used to improve the sensory properties of MJ products. The results obtained through this work can be applied to other fermented foods where salt is an important component. Use of solar salt instead of purified salt and bamboo salt can cause changes in microbial community of a specific food, which can positively affect the quality of the corresponding food.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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