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# Some Properties and Microbial Community Changes of Gul (Oyster) Jeotgal during Fermentation

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**Gul jeotgals (GJs) were prepared using solar salt aged for 3 years. One sample was fermented using starters, such as *Bacillus subtilis* JS2 and *Tetragenococcus halophilus* BS2-36 (each $10^6$ CFU/g), and another sample was fermented without starters for 49 days at 10℃. Initial counts of bacilli and lactic acid bacteria (LAB) in non-starter GJ were found to be $3.20 \times 10^2$ and $7.67 \times 10^1$ CFU/g on day 0, and increased to $1.37 \times 10^3$ and $1.64 \times 10^6$ CFU/g on day 49. Those of starter GJ were found to be $2.10 \times 10^5$ and $3.30 \times 10^7$ CFU/g on day 49, indicating the growth of starters. The pH values of GJ were 5.93 ± 0.01 (non-starter) and 5.92 ± 0.01 (starter) on day 0 and decreased to 5.78 ± 0.01 (non-starter) and 5.75 ± 0.01 (starter) on day 49. Amino-type nitrogen (ANN) production increased continuously during fermentation, and 407.19 ± 15.85 (non-starter) and 398.04 ± 13.73 (starter) mg% on day 49. Clone libraries of 16S rRNA genes were constructed from total DNA extracted from non-starter GJ on days 7, 21, and 42. Nucleotide sequences of *Escherichia coli* transformants harboring recombinant pGEM-T easy plasmid containing 16S rRNA gene inserts from different bacterial species were analyzed using BLAST. Uncultured bacterium was the most dominant group and Gram – bacteria such as *Acidovorax* sp., *Afipia* sp., and *Variovorax* sp. were the second dominant group. *Bacillus amyloliquefaciens* (day 7), *Bacillus velezensis* (day 21 and 42), and *Bacillus subtilis* (day 42) were observed, but no lactic acid bacteria were detected. *Acidovorax* and *Variovorax* species might play some role in GJ fermentation. Further studies on these bacteria are necessary.**

**Keywords:** **Gul jeotgal, *Tetragenococcus halophilus, Bacillus subtilis,* 16S rRNA clone library**

## Introduction

Gul (Oyster, *Crassostrea gigas*) is massively cultivated at the southern coast regions of Korean Peninsula. Gul is mostly consumed as raw material or an important ingredient of kimchi. Gul jeotgal is a common side dish for Korean cuisine. Eoriguljeot is the most popular type of gul jeotgal, which is prepared by mixing gul with salt, and red pepper powder, and fermented for just a few days before consumption [1]. Gul jeotgal with salt only is also prepared, and the salt concentration is around 20% (w/w) and fermented for a month [1]. On the contrary to its popularity, not many studies on gul jeotgal have been done. Just a few studies have been reported and they were on protein hydrolysis and production of flavoring agents during gul jeotgal fermentation [2, 3]. As an effort to increase oyster consumption throughout a year, development of novel processed products have been tried and these include oyster soup and spaghetti sauce [4, 5].

Literally no studies have been done so far on the microorganisms involved in gul jeotgal fermentation. It is unknown what microorganisms are major organisms during gul jeotgal fermentation and their effects on the quality of gul jeotgal. It is necessary to understand important microbial species, their growth, and their effects on gul jeotgal to produce high quality gul jeotgal

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in a consistant way. The purposes of this study were first to find out major bacterial species during gul jeotgal fermentation, and the second was to evaluate the effects of starters for gul jeotgal fermentation. In our previous work, gul jeotgal with high salinities (23% NaCl, w/v) were prepared and fermented for 24 weeks at 15℃ [6]. But growth of microorganisms including starter (*B. subtilis* JS2) was poor due to the high salinity. This and the recent trend demanding for low salt foods are the main reason for us to prepare gul jeotgal with lower salt concentration. In this work, gul jeotgal (GJ) with 9.1% NaCl concentration was prepared and fermented for 49 days at 10℃. Unlike previous work, *Tetragenococcus halophilus* BS2-36 was used as a starter together with previously used *B. subtilis* JS2. Some important property changes of GJ were measured during fermentation. Bacterial species were also determined by culture-independent method for non-starter GJ at day 7, 21, and 42.

## Materials and Methods

### Preparation of gul jeotgal

Gul (Oyster, *Crassostrea gigas*) was purchased from a local fish market (Korea) in February 2018. Immediately after purchase, gul was washed under running tap water, and stood for 10 min to remove excess water. Washed gul (2.0 kg) was mixed with solar salt aged for 3 years (Taepyung salt farm, Korea, NaCl 86.03%). The final salt concentration was 9.1% (w/w) in terms of NaCl content. *Bacillus subtilis* JS2 and *Tetragencoccus halophilus* BS2-36 were inoculated at $1 \times 10^6$ CFU/g for one sample (starter-GJ), and another sample was prepared without starter (non-starter GJ). GJ samples were fermented for 49 days at 10℃ and analyzed every 7 days during fermentation.

### Viable cell counting

Viable cells of bacilli, lactic acid bacteria (LAB), and yeasts were counted at every 7 days during fermentation. Twenty gram of GJ was mixed with 40 ml of peptone water (0.1%, w/v) and homogenized using a stomacher (stomacher®80, Seward, USA). Homogenate was filtered with a 3M homogenizer bag filter (3M, USA) and diluted serially with peptone water. MRS agar plates were used for LAB counting, LB agar plates for bacilli counting, and YM agar plates for yeast counting. Plates were incubated for 48 h at 37℃ for bacilli, and 72 h at 30℃ for LAB and yeasts counting. All measurements were done in triplicates and the mean values were used with standard deviations.

### pH and titratable acidity (TA) of GJ

Ten gram of homogenized GJ sample was mixed with 40 ml of distilled water, shaken for 1 h in a water bath (100 rpm, 30℃). Supernatant was obtained after centrifugation (4,000 ×*g*, 20 min). 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, and the amount of NaOH was used to calculate the amount of lactic acid (%). All measurements were done in triplicates and the mean values were used with standard deviations.

### Amino-type nitrogen (ANN), ammonia-type nitrogen (AMN) and volatile basic nitrogen (VBN) of GJ samples

ANN, AMN, and VBN of GJ samples were measured according to the methods described previously [7]. All measurements were done in triplicates and the mean values were used with standard deviations.

### Identification of bacterial species by a culture-independent method

Aliquots from non-starter GJ were collected at day 7, 21 and 42, and total DNA was extracted by using EZ-10 spin column soil DNA mini-prep kit (Bio Basic Inc., Canada). 16S rRNA genes were amplified by using universal primer pair, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCT TACGACTT-3'). PCR was done under the following conditions: initial denaturation at 94℃ for 5 min and then 40 cycles of 30 s at 94℃, 2 min at 57℃, and 2 min at 72℃. Amplified fragments were purified from agarose gels by using 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 by electroporation as described previously [8]. Transformants were selected on LB agar 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). For each sample, 30 colonies were randomly selected on LB agar plates, and plasmids were prepared

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for DNA sequencing. DNA sequences were determined at Cosmogenetech (Korea). BLAST program (National Center for Biotechnology Information) was used to find homologous sequences in the data library (http://www.ncbi.nlm.nih.gov).

## Results and Discussion

### Changes in the viable cell counts during fermentation

Yeasts were not detected from GJ samples until the end of fermentation (day 49). Bacilli were counted by spreading GJ samples onto LB agar plates. Bacilli counts of non-starter GJ were $3.20 \times 10^2$ CFU/g at day 0, and slightly increased to $1.37 \times 10^3$ CFU/g at day 49, showing 4.3 fold increase during fermentation (Table 1).

LAB counting was done by spreading GJ samples onto MRS agar plates. LAB counts of non-starter GJ were $7.67 \times 10^1$ CFU/g at day 0, and $1.64 \times 10^6$ CFU/g at day 49, showing significant increase (21,382 fold) during fermentation (Table 1). LAB count increased continuously during fermentation. Compared to bacilli, LAB count increased significantly. It was possible that mineral rich solar salt might encourage the growth of LAB. Minerals such as Ca, K, and Mg are present in significant concentrations in solar salt, and these minerals seemed to encourage the growth of LAB [9, 10]. This explanation seems reasonable considering that LAB, well-known fastidious organisms, exhibit very complex nutritional requirements for growth including many minerals [11].

Bacilli counts of starter GJ were $3.80 \times 10^6$ CFU/g at day 0, and reduced to $2.1 \times 10^5$ CFU/g at day 49 (Table 1). Bacilli counts decreased gradually during fermentation, and the final count was about 5.5% of initial count. LAB counts of starter GJ showed different results. LAB counts decreased gradually until day 21, but after day 21, increased slowly until the end of fermentation, and the final count was 10-fold higher than the initial count. Considering the initial inoculum size (each $10^6$ CFU/g), the results indicated that both starters adapted to the environments of GJ to some extents. But exuberant growth was not observed, and this was more apparent for *B. subtilis* JS2. Environments of GJ fermentation (10℃ and 9.1% NaCl) might not be ideal for *B. subtilis* JS2.

We previously prepared GJ samples with 23% NaCl concentration (w/w) and *B. subtilis* JS2 was used as a single starter ($1 \times 10^6$ CFU/g) [6]. Fermentation was done for 24 weeks at 15℃. Bacilli counts of non-starter and starter GJ were $1.50 \times 10^2$ CFU/g and $1.04 \times 10^3$ CFU/g, respectively, at 6 weeks. Bacilli count of starter GJ sample was less than 2 log scale than that from this work (day 42). In previous work, LAB were not detected until 8 weeks, and after 8 weeks, sporadically detected, and the highest number was 545 CFU/g [6]. High salinity of previous GJ samples was the reason for poor growth of bacilli and LAB. Jeotgal and other fermented foods are traditionally prepared with high salinities (20–30%, w/w) to prevent growth of spoilage microorganisms. These days, however, low salt foods are preferred because consumption of high salt foods is known to cause adverse health effects [12].

An important objective of this work was to test growth of *Bacillus* and LAB starters at 9% NaCl concentration because active growth of starters together with growth inhibition of many spoilage organisms were expected at this salt concentration. In this work, growth of LAB was confirmed, and survival of *B. subtilis* JS2 was also confirmed. But more studies are necessary on the identifica-

**Table 1. Changes in the viable cell numbers of bacilli and LAB of GJ samples (CFU/g) during fermentation.**

| Sample | | Fermentation period (Day) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 21 | 28 | 42 | 49 |
| Bacilli (CFU/g) | Starter | $3.80 \times 10^6$ ± 0.67 | $1.63 \times 10^6$ ± 0.02 | $1.20 \times 10^6$ ± 0.11 | $7.67 \times 10^5$ ± 0.12 | $3.27 \times 10^5$ ± 0.02 | $3.93 \times 10^5$ ± 0.05 | $2.10 \times 10^5$ ± 0.07 |
| | Non-starter | $3.20 \times 10^2$ ± 0.24 | $6.30 \times 10^2$ ± 0.07 | $5.87 \times 10^2$ ± 0.05 | $2.27 \times 10^2$ ± 0.04 | $6.40 \times 10^2$ ± 0.28 | $8.00 \times 10^2$ ± 0.03 | $1.37 \times 10^3$ ± 0.18 |
| LAB (CFU/g) | Starter | $3.33 \times 10^6$± 0.78 | $1.32 \times 10^6$ ± 0.06 | $1.26 \times 10^6$ ± 0.08 | $5.73 \times 10^5$ ± 0.09 | $6.32 \times 10^5$ ± 0.01 | $1.12 \times 10^7$ ± 0.05 | $3.30 \times 10^7$ ± 0.04 |
| | Non-starter | $7.67 \times 10^1$ ± 0.01 | $1.00 \times 10^2$ ± 0.07 | $1.40 \times 10^2$ ± 0.05 | $1.89 \times 10^3$ ± 0.12 | $1.00 \times 10^4$ ± 0.10 | $1.54 \times 10^5$ ± 0.08 | $1.64 \times 10^6$ ± 0.24 |

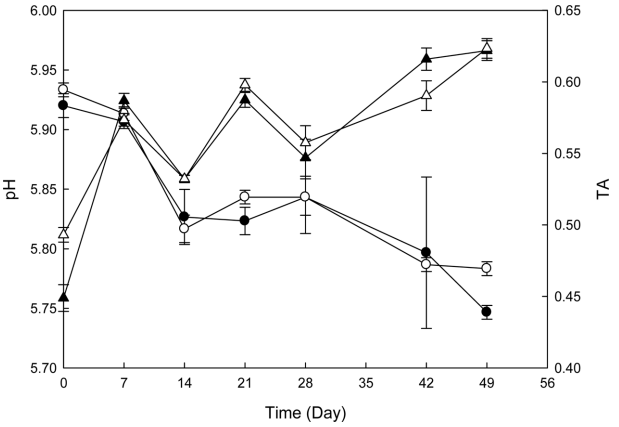


**Fig. 1. Changes in pH and titratable acidity of GJ samples during fermentation.** ●, starter GJ (pH); ○, non-starter GJ (pH); ▲, starter GJ (TA); △, non-starter GJ (TA).

tion of LAB species growing during GJ fermentation including *T. halophilus* BS2-36. Testing other *Bacillus* strains is also necessary. In addition, lower NaCl concentration such as 5% should be tested for GJ fermentation. Lowering NaCl concentration affects not only growth of starters but also undesirable spoilage organisms. It also affects the taste and flavor of GJ. Fermentation temperature and time also affect progress of fermentation and the final quality of GJ. All these factors are carefully optimized if high quality GJ products are produced.

### pH and titratable acidity of GJ

pH of GJ samples decreased gradually during fermentation (Fig. 1). At day 0, pH of non-starter and starter GJ were 5.93 ± 0.01 and 5.92 ± 0.01, respectively. At day 49, pH of non-starter and starter GJ were 5.78 ± 0.01 and 5.75 ± 0.01, respectively. Starter GJ showed slightly lower pH.

TA values of GJ samples increased gradually during fermentation (Fig. 1). The initial values were 0.49 ± 0.01 for non-starter GJ, and 0.45 ± 0.01 for starter GJ. At day 49, TA of starter and non-starter GJ was the same, 0.62 ± 0.01. pH and TA values of fermented foods are affected by organic acids such as lactic acid produced by LAB during fermentation [13].

### Amino type nitrogen (ANN), ammonia type nitrogen (AMN), and volatile basic nitrogen (VBN) of GJ samples

ANN of GJ samples were measured during fermentation (Fig. 2A). ANN content of a fermented food is related with the degree of protein hydrolysis of raw materials. Proteases from either food materials or microorganisms degrade proteins of raw materials, generating

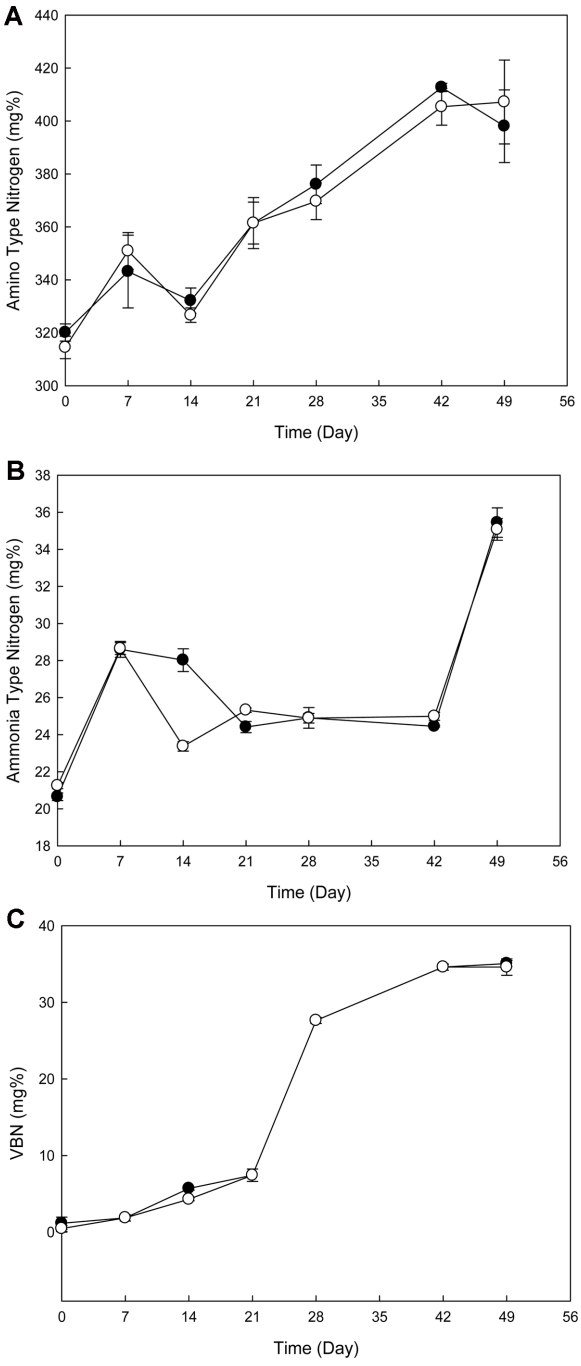


**Fig. 2. Changes in amino-type nitrogen (A) ammonia-type nitrogen (B) and volatile basic nitrogen (C) of GJ samples during fermentation.** ●, starter GJ; ○, non-starter GJ.

peptides and amino acids, contributing to the development of unique flavor, taste, and texture of fermented foods [14]. Immediately after jeotgal preparation, ANN contents were 314.49 ± 4.23 mg% for non-starter GJ and 320.13 ± 3.23 mg% for starter GJ. ANN contents increased continuously during fermentation, and the final values were 407.19 ± 15.85 mg% for non-starter GJ and 398.04 ± 13.73 mg% for starter GJ.

AMN contents of GJ samples were increased until day 7, and then reduced slightly and remained at similar levels until day 42, and then increased rapidly (Fig. 2B). No significant differences were observed between non-starter and starter GJ except day 14. At day 49, AMN contents were 35.44 ± 0.79 mg% for starter GJ and 35.07 ± 0.58 mg% for non-starter GJ. VBN is lower basic nitrogen compounds with volatility, such as ammonia, and trimethylamine [15]. Immediately after preparation (day 0), VBN contents were very low, 1.86 ± 0.40 mg% for both starter and non-starter GJ (Fig. 2C). VBN increased continuously, especially rapidly increased between day 21 and day 28. The final values were 34.59 ± 1.06 mg% for non-starter GJ and 35.06 ± 0.40 mg% for starter GJ. No significant differences were observed between starter and non-starter GJ.

**Table 2. Identification of clone library prepared from non-starter GJ at day 7, 21 and 42 by a culture-independent method.**

| Day | 16S rRNA gene sequencing (NCBI Accession No.) | Similarity (%) |
|---|---|---|
| 7 | Uncultured bacterium (HM141889.1) 8 | 99 |
| | Uncultured bacterium (GU455124.1) 2 | 99 |
| | Uncultured bacterium (HE574374.1) | 98 |
| | Uncultured bacterium (HE647148.1) | 100 |
| | *Afipia birgiae* (NR_025117.1) 4 | 99 |
| | *Afipia* sp. (EU130950.1) 2 | 99 |
| | *Bradyrhizobium* sp. (AB681389.1) 3 | 99 |
| | *Bradyrhizobium* sp. (KY445662.1) | 99 |
| | *Bradyrhizobium* sp. (MG798777.1) | 99 |
| | *Bradyrhizobium elkanii* (LC386884.1) 2 | 99 |
| | *Bradyrhizobium japonicum* (LC386886.1) | 99 |
| | *Stenotrophomonas maltophilia* (CP029773.1) | 96 |
| | *Alphaproteobacteria* bacterium (KY053157.1) | 99 |
| | *Bacillus amyloliquefaciens* (MH144310.1) 2 | 99 |
| 21 | Uncultured bacterium (GQ388939.1) 8 | 98 |
| | Uncultured bacterium (HM141889.1) | 100 |
| | *Variovorax* sp. (MH698893.1) 8 | 100 |
| | *Variovorax* sp. (MH699131.1) | 99 |
| | *Acidovorax* sp. (MH512862.1) 2 | 99 |
| | *Acidovorax* sp. (AY093698.1) | 99 |
| | *Afipia birgiae* (NR_025117.1) | 99 |
| | *Afipia* sp. (EU130950.1) | 99 |
| | *Ralstonia* sp. (HE575954.1) | 98 |
| | *Ralstonia* sp. (LC385700.1) | 100 |
| | *Comamonas* sp. (MH698867.1) | 100 |
| | *Pseudomonas* sp. (FJ976073.1) | 99 |
| | *Sphingopyxis taejonensis* (KX682024.1) | 99 |
| | *Bacillus velezensis* (KY427069.1) 2 | 99 |
| 42 | Uncultured bacterium (HM141889.1) 24 | 99 |
| | *Variovorax* sp. (MH698893.1) 2 | 100 |
| | *Burkholderia* sp. (MH561728.1) | 99 |
| | *Bacillus velezensis* (KY427069.1) 2 | 99 |
| | *Bacillus subtilis* (AM110924.1) | 99 |

### Bacterial communities of GJ samples

Uncultured bacterium was the most dominant group of clone libraries at day 7, 21, and 42 (Table 2). Uncultured bacterium occupied 40% (12 out of 30) at day 7 followed by *Bradyrhizobium* sp. (26.7%, 8 out of 30) and *Afipia* sp. (20%, 6 out of 30). *Bacillus amyloliquefaciens* was detected once (3.3%). *Stenotrophomonas maltophilia,* and *Alphaproteobacteria* bacterium were also detected each once (3.3%).

At day 21, *Variovorax* sp. was the most dominant group (30%, 9 out of 30) together with uncultured bacterium. *Acidovorax* sp. was detected 10% (3 out of 30), and *Afipia* sp., *Ralstonia* sp., and *B. velezensis* were each detected 6.7% (2 times), respectively. Each of *Comamonas* sp., *Pseudomonas* sp., and *Sphingopyxis taejonensis* was detected 3.3% (1 out of 30), respectively.

*Variovorax* sp. are widely present at various environments of soil and water including ocean sediment near the Arctic [16, 17]. They possess diverse metabolic capacities, and being considered promising for bioremediation [18]. Like *Variovorax* sp., *Acidovorax* sp. are isolated from various soil and water environments, and being studied for their possible roles for degrading pollutants [19]. *Bacillus velezensis* and *B. amyloliquefaciens* are widely present at various fermented foods such as jeotgal and meju [20, 21]. Most *Bacillus* sp. are known to tolerate NaCl up to 15% [22]. At day 42, uncultured bacterium was the most dominant group, occupying 80% (24 out of

30) of clone library. *Variovorax* sp. and *B. velezensis* were each detected 6.7% (2 out of 30). *Burkholderia* sp. and *B. subtilis* were each detected once (3.3%).

Majority of identified species belong to Gram – bacteria which are widely distributed in nature including soil and water, and possess the abilities of degrading diverse compounds. Marine environments are also the places where these Gram – bacteria proliferate. Gram + bacteria were not detected except *Bacillus* sp., and this was unexpected since *Tetragenococcus* sp. such as *T. halophilus* has been reported as the major group in fish sauce and jeotgal with high salinities [23–25]. Considering the fermentation conditions (9% NaCl and 10℃), detection of *Tetragenococcus* sp. was expected. In our previous study, *T. halophilus* strains were isolated from myeolchi jeotgal (23% NaCl, w/v), and they showed good growth in MRS broth with 10% NaCl at 15℃ [6]. It is not clear why *Tetragenococcus* sp. were not detected from GJ. Probably, they grew slowly at 10℃, and present in small numbers until day 42, which prevented successful amplification of their 16S rRNA genes. Another explanation is that gul (oyster)-jeotgal is not a good environment for *Tetragenococcus* sp. The failure for detecting LAB might be also due to the inherent biases of culture-independent method such as different efficiencies of primers and DNA extraction method for different bacteria [26]. Cultural method should be tried together with culture-independent method for future works on microbial community analysis for GJ.

Through this work, we showed for the first time some members of microbial community of GJ under a specific conditions (NaCl 9.1% and 10℃) by 16S rRNA gene clone library. We also showed a possibility that some gram – bacteria such as *Acidovorax* and *Variovorax* species might play some roles for GJ fermentation. Since nothing is known for the roles of these Gram – bacteria for GJ fermentation, future studies are necessary. In addition, efforts to isolate bacilli and LAB with desirable properties as starters should be continued. These efforts will eventually lead to production of high-quality gul jeotgal in a consistant way.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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