

Properties of Gul Jeotgal (Oyster Jeotgal) Prepared with Different Types of Salt and *Bacillus subtilis* JS2 as Starter

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Gul (oyster) *jeotgals* (GJs) were prepared using different types of salt (23%, w/v): purified salt, solar salt aged for 3 years, and bamboo salt crystalized 3 times. One set of GJs was fermented with *Bacillus subtilis* JS2 (10^6 CFU/g), while the other GJ set was fermented without starter. During fermentation for 24 weeks at 15°C, the starter GJs showed 10-fold higher bacilli counts than the no-starter GJs, where the maximum bacilli count was 8×10^3 CFU/g. All 28 bacilli strains isolated from the 6-week GJs were identified as *B. subtilis* by using a RAPD-PCR, indicating that some of the *B. subtilis* JS2 cells remained viable. Lactic acid bacteria (LAB) and yeasts were present at low levels, 10^1 – 10^2 CFU/g. LAB with protease activities isolated from 10-week samples were identified as *Enterococcus* species. The isolates obtained at 16 weeks were all *Staphylococcus* species. The GJs with bamboo salt showed higher pH and lower titratable acidity (TA) values than the other GJs due to the strong alkalinity of bamboo salt. The amino-type nitrogen in the GJs increased slowly during the fermentation. At 24 weeks, the GJs with purified salt showed the highest amino-type nitrogen (412–430 mg%), followed by the GJs with solar salt (397–406 mg%) and GJs with bamboo salt (264–276 mg%). Meanwhile, the GJs with bamboo salt showed the highest ammonia-type N (63–67 mg%), followed by the GJs with purified salt (49 mg%) and solar salt (48 mg%).

Keywords: Gul jeotgal, solar salt, bamboo salt, purified salt

Introduction

Jeotgals are traditional Korean fermented and salted sea foods that are produced using various fish, fish eggs, fish intestines, and shellfish [1, 2]. The most popular jeotgals are saeu (shrimp) jeotgal and myeolchi (anchovy) jeotgal, which are fermented for several months or even years before consumption [2]. In contrast, gul (oyster, *Crassostrea gigas*) jeotgal, which is also popular, can be consumed within a couple of weeks. Gul jeotgal (GJ) is prepared by mixing fresh gul with salt and seasonings,

including red pepper powder, garlic, and scallions [2]. While a few studies have already examined the changes in the free amino acids and nucleotides during GJ fermentation [3, 4], no studies have yet investigated the microbiota involved in GJ fermentation, probably due to the short fermentation time for GJ. Accordingly, this study prepared GJ samples using 3 different types of salt (purified salt, solar salt, and bamboo salt) to check whether the different salt types affected the quality of the GJ. In previous studies by the current authors, different fermented foods (kimchi, doenjang, and myeolchi jeotgal) were also prepared using different types of salt, and it was shown that the growth of lactic acid bacteria (LAB) and yeasts was significantly affected by the salt type [5–7]. In particular, the growth of yeasts was

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encouraged in the presence of purified salt, whereas the growth of LAB was encouraged with solar salt and bamboo salt. In the present study, *Bacillus subtilis* JS2, an isolate from saeu jeotgal with strong fibrinolytic activity, was also added as a starter to one set of GJ samples, since the addition of a starter is believed to accelerate the fermentation process and improve the overall quality of jeotgals [8]. The fermentation of jeotgals is currently carried out using only natural microbiota, which are significantly affected by the type of raw material, salinity of the jeotgal, and temperature of the fermentation, resulting in jeotgals with varying qualities. The present study also examined the possibility of long-term fermentation of GJ without seasoning, except for salt. The sensory properties of long-term fermented GJ were expected to be different from those of short-term fermented and seasoned GJ. The GJ sample properties were measured, including a viable cell count of bacilli, lactic acid bacteria (LAB), and yeasts, during the fermentation of the GJ samples up to 24 weeks at 15 °C. A RAPD (randomly amplified polymorphic DNA)-PCR was used to identify *B. subtilis* JS2 in the GJ samples. It is hoped that the results will be useful for producing long-term fermented GJ and predicting changes in the microbiota during fermentation.

Materials and Methods

Preparation of gul jeotgals

Gul (Oyster, *Crassostrea gigas*) was purchased from a local fish market (Tongyeong, Gyeongnam, Korea) in December, 2016. Right after purchasing, the gul was washed under running tap water, and left for 10 min to remove any excess water. Next, 9 kg of gul was mixed with salt, where NaCl concentration in the GJ was adjusted to 23% (w/w) by adding different amounts of each salt: 2,091 g (23%) for purified salt (PS, Hanju, Korea, 2016, NaCl 99%); 2,190 g (23%) for solar salt (SS, Taepyung salt farm, Korea, aged for 3 years, NaCl 94.54%); and 2,190 g (23%) for bamboo salt (BS, Insanga, Hamyang, Gyeongnam, Korea, melted and recrystallized 3 times, NaCl 94.54%). One set of GJs included *Bacillus subtilis* JS2 inoculated at 1×10^6 CFU/g (starter GJs), while the other set was prepared without any starter (no-starter GJs). All 6 GJs were fermented for 24 weeks at 15 °C, and analyzed every 2 weeks during

the fermentation.

Viable cell counting

Twenty grams of each GJ was mixed with 20 ml of peptone water (0.1%, w/v) and homogenized using a stomacher (stomacher[®]80, USA). The homogenate was filtered using a bag filter (Interscience, France) and diluted serially with peptone water. The diluted samples were then spread on MRS agar (Accumedia, USA) plates containing cycloheximide (50 µg/ml) for LAB counting, Luria-Bertani (LB, Accumedia) plates for bacilli counting, and Yeast-Mold agar (YM, BD Difco, USA) plates with rifampicin (50 µg/ml) for yeast counting. The plates were incubated for 48 h at 37 °C for the bacilli counting, and for 96 h at 30 °C for the LAB and yeast counting.

Isolation of isolates with proteolytic activities

The GJ samples at 10 and 16 weeks were plated on MRS agar plates (Neogen, USA) containing cycloheximide (50 µg/ml), and colonies selected after incubation. The selected colonies were inoculated into an MRS broth (Accumedia) for 48 h at 30 °C. Aliquots (2 µl each) of each culture were spotted on MRS agar plates containing 0.006% BCP (bromocresol purple) and 1% CaCO₃, and the plates incubated for 48 h at 30 °C. Colonies showing a yellow color and clear zone were inoculated into an MRS broth and grown for 48 h at 30 °C. Two µl of each culture was then spotted onto an MRS agar plate containing 1% skim milk, and the plates incubated for 48 h at 30 °C.

Identification of isolates

To identify the isolates, 16S rRNA gene sequencing was performed using universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGY-TACCTTGTTACGACTT-3'), as described previously [9].

RAPD-PCR of selected bacilli colonies

The GJ samples at 6 weeks were spread on LB agar plates with 5% NaCl, and the plates incubated until bacilli colonies appeared. Selected colonies (8 from Purified Salt (PS)GJ, and 10 from Solar Salt (SS)GJ and Bamboo Salt (BS)GJ) were inoculated into 5 ml of LB and grown for 18 h at 37 °C. The chromosomal DNA was prepared using phenol-chloroform extraction. The RAPD-PCR was conducted using an S30 primer (5'-GTGATCGCAG-3') as the single primer, while the other

conditions were the same as described previously [10].

pH and titratable acidity (TA) measurements

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

Amino-type nitrogen, ammonia-type nitrogen, and volatile basic nitrogen measurements

The amino-type nitrogen (ANN), ammonia-type nitrogen (AMN), and volatile basic nitrogen (VBN) in the GJ samples were all measured using methods described previously [5].

Results and Discussion

Changes in viable counts of bacilli, LAB, and yeasts during GJ fermentation.

The viable cell counts of bacilli, LAB, and yeasts were measured during 24 weeks of fermentation (Table 1). The bacilli counts in the starter GJ samples ranged from 1.0 to 7.9×10^3 CFU/g, which was 10 times higher than those in the no-starter GJ samples ($1.0\text{--}9.7 \times 10^2$ CFU/g). When considering the inoculum size of *B. subtilis* JS2 (10^6 CFU/g), the results indicated that most *B. subtilis* JS2 cells were apparently killed soon after addition. The *B. subtilis* JS2 was initially isolated from saeu jeotgal and able to grow in an LB broth with 20% (w/v) NaCl (results not shown). This *B. subtilis* strain also exhibits strong fibrinolytic activity. Thus, it was expected that *B. subtilis* JS2 would grow in GJ with 23% NaCl and contribute to the GJ fermentation. However, *B. subtilis* JS2 did not grow, although some cells did remain viable during the 24 weeks of fermentation. A RAPD-PCR was conducted for selected colonies obtained from the starter GJ samples at 6 weeks, as described in the methods section. All the colonies, except for S8, showed the same RAPD-PCR profile as that for *B. subtilis* JS2, producing 0.5 kb and 0.88 kb fragments (Fig. 1). In the case of S8, the band intensities were too low to make a conclusion. Thus, all the colonies showed the same morphology and strong fibrinolytic activities like *B. subtilis* JS2. While a

Table 1. Changes in the viable cell numbers of bacilli, yeasts and LAB during fermentation.

Sample	Fermentation period (week)													
	0	2	4	6	8	10	12	14	16	18	20	22	24	
Bacilli (CFU/g)	PS	1.30×10^2	^a	9.70×10^2	3.5×10^1	2.15×10^2	2.10×10^2	4.50×10^2	1.57×10^2	3.18×10^2	5.18×10^2	4.15×10^2	3.55×10^2	1.90×10^2
	SS	2.50×10^2	2.50×10^1	3.70×10^2	1.50×10^2	1.90×10^2	2.00×10^2	1.00×10^2	4.35×10^2	2.64×10^2	3.35×10^2	4.65×10^2	2.60×10^2	5.10×10^2
	BS	3.80×10^2	-	5.00×10^2	1.60×10^2	3.90×10^2	1.95×10^2	2.55×10^2	3.58×10^2	3.08×10^2	3.67×10^2	3.35×10^2	3.25×10^2	2.43×10^2
	PSB	3.00×10^3	1.75×10^3	1.35×10^3	1.82×10^3	2.49×10^3	2.41×10^3	2.61×10^3	2.42×10^3	3.28×10^3	3.10×10^3	3.18×10^3	2.97×10^3	3.09×10^3
	SSB	4.50×10^3	1.55×10^3	1.50×10^3	1.04×10^3	2.27×10^3	2.37×10^3	1.96×10^3	2.39×10^3	3.35×10^3	2.81×10^3	2.89×10^3	3.29×10^3	3.26×10^3
	BSB	2.00×10^3	1.00×10^3	1.52×10^3	2.22×10^3	2.74×10^3	2.68×10^3	4.75×10^3	3.40×10^3	4.69×10^3	7.87×10^3	3.55×10^3	3.29×10^3	2.50×10^3
Yeast (CFU/g)	PS	-	-	-	-	-	-	-	2.00×10^1	-	1.25×10^2	1.10×10^2	1.00×10^1	-
	SS	-	-	-	-	-	-	-	1.00×10^1	-	-	-	1.00×10^1	1.00×10^1
	BS	-	-	-	-	-	-	-	3.50×10^1	-	-	-	-	1.00×10^1
	PSB	-	-	-	-	-	-	-	-	-	-	-	1.00×10^1	-
	SSB	-	-	-	-	-	-	-	-	-	-	-	-	1.00×10^1
	BSB	-	-	-	-	-	-	-	-	4.50×10^1	-	-	2.50×10^1	-
LAB (CFU/g)	PS	-	-	-	-	-	9.00×10^1	-	-	6.80×10^1	-	-	1.00×10^1	1.00×10^1
	SS	-	-	-	-	-	1.00×10^1	-	1.00×10^1	1.50×10^1	-	9.50×10^1	1.00×10^1	1.00×10^1
	BS	-	-	-	-	-	3.30×10^2	-	-	-	-	-	5.67×10^1	4.00×10^1
	PSB	-	-	-	-	-	-	-	-	-	4.00×10^1	4.00×10^2	2.25×10^2	-
	SSB	-	-	-	-	-	-	-	-	1.00×10^1	4.50×10^1	5.45×10^2	1.00×10^1	-
	BSB	-	-	-	-	-	-	-	-	3.55×10^2	-	4.98×10^2	-	1.00×10^1

^aViable cells (bacilli, yeast and lactic acid bacteria) were not detected.

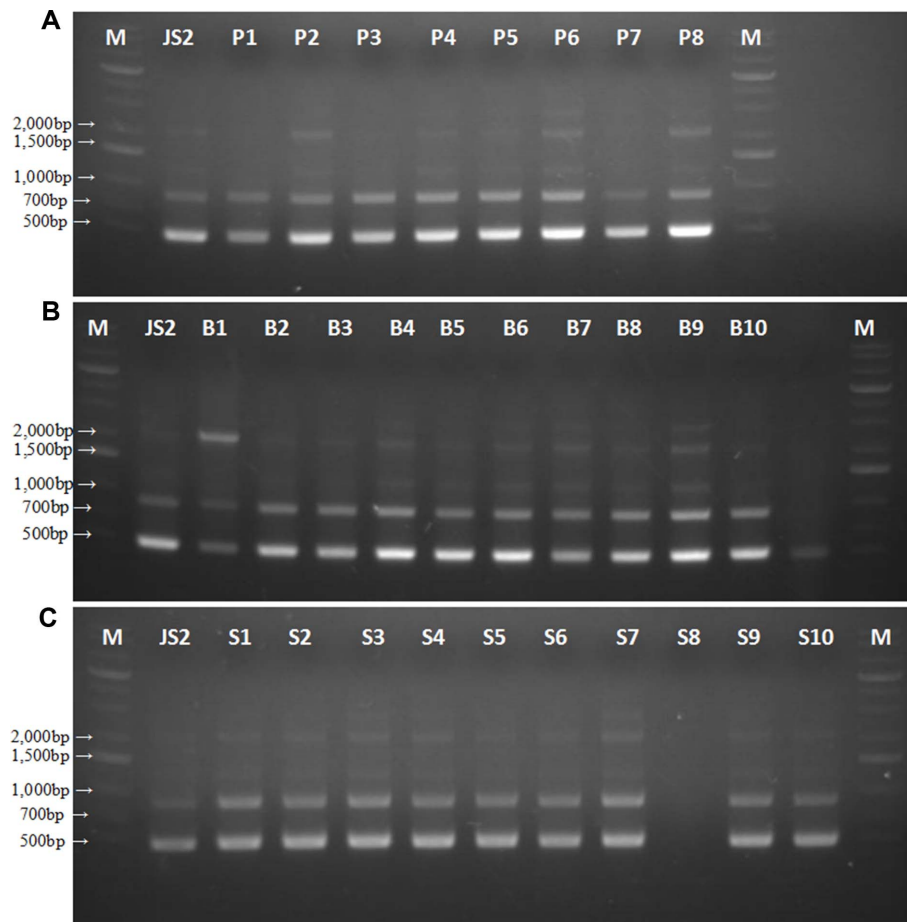


Fig. 1. RAPD-PCR profiles of isolates and JS2. RAPD-PCR results of JS2 and isolates from different Jeotgals. A, PS (Purified salt) GJ; B, BS (Bamboo salt) GJ; C, SS (Solar salt) GJ. M, GeneRuler 1 kb Plus DNA Ladder (Sigma-Aldrich, D-0428); JS2, *Bacillus subtilis* JS2; P1-P8, isolates from PSGJ; B1-B10, isolates from BSGJ; S1-S10, isolates from SSGJ. GJ samples were taken at 6 weeks.

RAPD-PCR is a rapid and reliable method for identifying *Bacillus* species identification, 16S rRNA gene sequencing is unable to discriminate closely related *Bacillus* species [10]. When considering that the *B. subtilis* JS2 viable cell counts in the starter GJ samples were 10 times higher than those in the no-starter GJ samples, it is reasonable to assume that some *B. subtilis* JS2 cells survived during the GJ fermentation and were the main *Bacillus* species. Yet, since there was no growth of *B. subtilis* JS2, the effects of the starter on the fermentation of the GJ samples remain unknown. Thus, inoculating *B. subtilis* JS2 into GJ with a lower NaCl concentration will be needed to understand the role of *B. subtilis* JS2 in GJ fermentation. Lower bacilli counts have also been observed during the fermentation of myeolchi jeotgals (20% NaCl, w/w) [5].

The LAB and yeast counts in the GJs were lower than the bacilli counts throughout the fermentation period, ranging from 10^1 to 10^2 CFU/g, and no significant differences were observed among the GJ samples with the different salt types. Most of the LAB and yeasts were unable to grow at the 23% NaCl concentration. Lower yeast counts have also been observed in myeolchi jeotgals [5] and saeu jeotgals prepared with 3 different types of salt (25%, w/w) [11], although, unlike GJs, LAB were only isolated from saeu jeotgals during the first 10 weeks and not isolated at 12 weeks and thereafter [11].

Identification of isolates showing proteolytic activities

From the GJ samples at 10 weeks, 2 colonies showing proteolytic activities were selected from the BSGJ and one from the PSGJ. BLAST analyses of their 16S rRNA

Table 2. Identification of isolates with proteolytic activities at 10 weeks.

Isolates	Species	% Identity
BS1	<i>Enterococcus durans</i>	99% ^a
	<i>Enterococcus thailandicus</i>	99%
BS11	<i>Enterococcus faecium</i>	99%
	<i>Streptococcus</i> sp.	99%
	<i>Enterococcus hirae</i>	99%
PS2	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus warneri</i>	99%
	<i>Staphylococcus pasteurii</i>	99%

^a1,400 nucleotides from 16S rRNA genes were sequenced and analyzed by BLAST.

genes showed that the two colonies from the BSGJ were most likely to be *Enterococcus* species, while the colony from the PSGJ was a *Staphylococcus* species (Table 2). The two *Enterococcus* strains (BS1 and BS11) grew well in an MRS broth with 8% NaCl, yet grew poorly with 10% NaCl, meanwhile the *Staphylococcus* strain (PS2) was able to grow with 20% NaCl. All the colonies from the PSGJ at 16 weeks were identified as staphylococci (Table 3). These results agree well with other studies. Fukui *et al.* prepared fish sauce (15% NaCl, w/w) using deep sea smelt (*Glossanodon semifasciatus*) that was stored at room temperature [12]. In this case, staphylococci were identified as the most dominant organisms until 4 weeks, and then tetragenococci became the most dominant after 6 weeks [12]. Enterococci were also isolated as minor members when using a PGA (plate count agar) plate without salt. Moreover, other studies have reported staphylococci and tetragenococci as the most dominant organisms in jeotgals and fish sauces with high salinities [12–15]. Interestingly, in the current study, no tetragenococci were isolated from the GJ samples, despite several attempts. Thus, *tetragenococcus* species may have problems growing on GJ. Each group of LAB has different growth requirements, and oysters by themselves may not provide the necessary nutrients for the growth of *Tetragenococcus* species. *Bacillus* species were previously reported as the most dominant organisms when plating ojingeo-jeotgal samples onto various culture media [16], although the average salt concentration of 6.7% was much lower than that in the current study. Among 121 isolates, 86 (71%) were identified as *Bacillus* species, including *B. methylotrophicus* (12 iso-

Table 3. Identification of isolates with proteolytic activities at 16 weeks.

Isolates	Species	% Identity
PS1	<i>Staphylococcus epidermidis</i>	100% ^a
PS4	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
PS5	<i>Staphylococcus epidermidis</i>	100%
PS7	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
PS8	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
PS12	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
PS14	<i>Staphylococcus</i> sp.	100%
	<i>Staphylococcus epidermidis</i>	100%
	<i>Staphylococcus caprae</i>	100%
	<i>Bacilli bacterium</i>	100%
PS15	<i>Staphylococcus epidermidis</i>	99%
	<i>Staphylococcus</i> sp.	99%
	<i>Bacilli bacterium</i>	100%
	<i>Staphylococcus caprae</i>	100%

^a1,400 nucleotides were sequenced and analyzed by BLAST.

lates), *B. tequilensis* (10), *B. subtilis* (9), *B. safensis* (7), *Bacillus aerius* (5), *B. amyloliquefaciens* (4), and other species.

As *Staphylococcus* species are a major group, growing well at high salinities and commonly isolated from various jeotgals, certain staphylococci strains could be considered as possible starters for jeotgals if the strains are proven to be safe [17]. In the present study, the *Staphylococcus* species from the PSGJ samples at 16 weeks were all identified as either *Staphylococcus epidermidis* or *Staphylococcus caprae*.

pH and TA measurements

Immediately following the GJ preparation, the pH val-

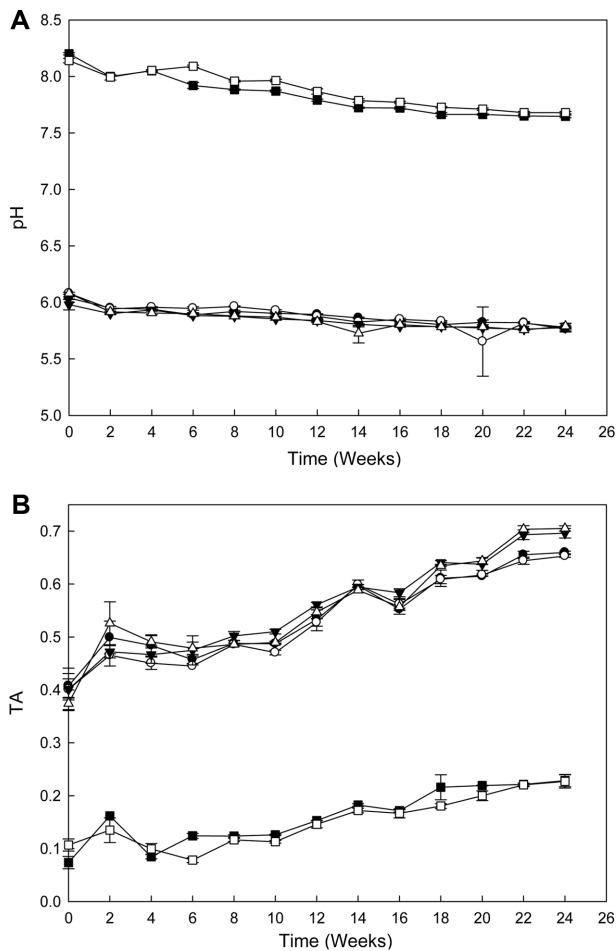


Fig. 2. Changes in pH (A) and TA (B) of GJ samples. ●, PSGJ (starter); ○, PSGJ (non-starter); ▼, SSGJ (starter); △, SSGJ (non-starter); ■, BSGJ (starter); □, BSGJ (non-starter).

ues for the BSGJs (both starter and no starter) ranged from 8.14 to 8.20, which were higher than those for the other GJ samples, which ranged from 5.98 to 6.08 (Fig. 2). This was due to the high alkalinity of the BS [18]. Plus, as the fermentation progressed, the pH values for the GJ samples slowly decreased, reaching 7.65–7.68 for the BSGJs and 5.76–5.79 for the other GJs at 24 weeks. The PS and SS GJs showed very similar pH values throughout the fermentation period.

The TA values for the BSGJs ranged from 0.07 to 0.11 immediately after preparation (0 weeks), which were lower than those for the other GJs (0.37–0.41). However, the TA values gradually increased, reaching 0.23 for the BSGJs and 0.65–0.71 for the other GJs at 24 weeks. The SSGJ samples showed the highest TA values. As expected, the BSGJs (both starter and no starter) exhib-

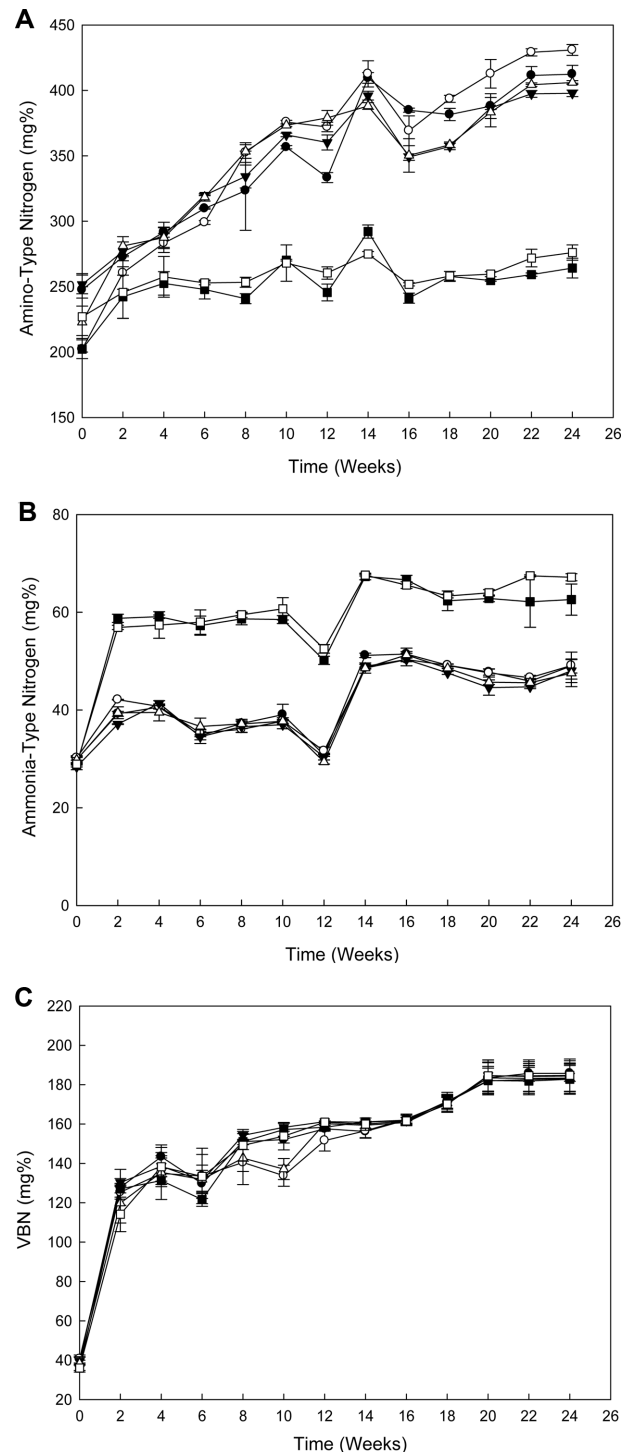


Fig. 3. Changes in ANN (A), AMN (B), and VBN (C) of GJ samples. ●, PSGJ (starter); ○, PSGJ (non-starter); ▼, SSGJ (starter); △, SSGJ (non-starter); ■, BSGJ (starter); □, BSGJ (non-starter).

ited higher pH values and lower TA values than the other GJ samples, which was caused by the higher pH of

the bamboo salt [19]. The decrease in the pH and increase in the TA values proceeded slowly and gradually due to the poor growth of LAB and bacilli under the high salt concentration. For the same reason, no significant differences were observed between the starter and no-starter GJs.

ANN, AMN, and VBN contents

The ANN, AMN, and VBN values of the GJ samples were also measured (Fig. 3). Immediately after GJ preparation (0 weeks), the ANN values ranged from 202.16 to 251.67 mg%, where the BSGJ with the starter showed the lowest value and the SSGJ with the starter showed the highest value. As the fermentation progressed, the ANN value showed a continuous increase in all the samples. The BSGJs (starter and no starter) showed the lowest values throughout the fermentation period. All the GJ samples, except for the no-starter SSGJ, showed a decrease of ANN at 12 weeks, followed by a sharp increase at 14 weeks. The PSGJs showed the highest ANN at 24 weeks, with 430.91 ± 4.17 mg% for the no-starter GJ and 412.32 ± 6.80 mg% for the starter GJ. For the SSGJs, the ANN contents were 406.00 ± 1.43 mg% and 397.76 ± 2.47 mg% for the no starter and starter, respectively. Notwithstanding, the addition of the starter did not cause the increased ANN, which was due to the poor growth of *B. subtilis* JS2, as mentioned earlier.

Immediately after GJ preparation (0 weeks), the AMN values were all very similar (28.38–30.19 mg%). However, after 2 weeks, the BSGJs showed significantly higher AMN values than the other GJs. This result was different from the ANN values, where the BSGJs showed lower values. It remains unclear why the BSGJ samples showed higher AMN values. In the case of myeolchi (anchovy) jeotgal fermentation, jeotgal prepared with bamboo salt showed higher ANN and AMN values than jeotgals prepared with SS and PS (20%, salt) [5]. Similar to the ANN values, the AMN values for the GJ samples gradually increased throughout the fermentation. Plus, similar to the ANN values, the AMN values also decreased sharply between 10 and 12 weeks, and then increased sharply until 14 weeks. After 14 weeks, the AMN values gradually decreased. The final AMN values ranged between 47.55 and 67, a 2-fold increase from the initial values. No significant differences

were observed between the starter and no-starter GJs.

The VBN values ranged from 35.99 to 40.63 mg% immediately after GJ preparation (0 weeks), followed by a sharp increase until 2 weeks, and then a gradual increase until the end of the fermentation. All the GJ samples showed similar VBN values throughout the fermentation. The starter and no-starter GJ samples also showed quite similar values, and no differences were observed among the samples with different salt types.

Under the conditions tested in this study, microorganisms with strong proteolytic activities were not dominant, which caused fewer changes in the ANN, AMN, and VBN contents when compared with other jeotgals. This may have been partly due to the properties of the oysters themselves. No putrefaction of the GJ samples was apparent after 24 weeks at 15°C, and no off-flavors were generated. The *B. subtilis* JS2 cells showed no proliferation and no noticeable differences were observed between the starter and no-starter GJs. Albeit, some *B. subtilis* JS2 cells were detected, indicating a possibility that they might grow quickly at lower salt concentrations. Thus, further studies are needed to determine the effects of *B. subtilis* JS2 at lower salt concentrations. Moreover, enterococci and streptococci were isolated, and these species likely played specific roles during the GJ fermentation. Notwithstanding, this study showed that GJ could be successfully fermented for a long time (24 weeks) without putrefaction, much longer than the usual period of 2–3 weeks. More detailed studies on the qualities of GJ, including its sensory properties and microbial communities, are needed in the future.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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