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Characteristics of Doenjang (Soybean Paste) Fermented with Multiple Starters Including *Tetragenococcus halophilus*

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Tetragenococcus halophilus **CY54, an isolate from jeotgal, grows best in media with 5% NaCl and can grow at 18% and higher salt concentration. Three different doenjang samples were prepared with multiple starters including *T. halophilus* CY54. TBZA doenjang was prepared with *T. halophilus*, *Bacillus subtilis*, *Zygosaccharomyces rouxii* and *Aspergillus oryzae*. BZA doenjang was prepared with the same 3 starters except *T. halophilus*. KACC doenjang was prepared with a single starter, *B. subtilis* KACC16750. During 16 weeks of fermentation at 25℃, the viable counts were maintained in the range of 7-8 log CFU/g in all 3 samples. As fermentation progressed, pH decreased and titratable acidity (TA) gradually increased. Crude protein contents decreased slightly. TBZA doenjang showed higher amino-type nitrogen (ANN) and volatile basic nitrogen (VBN) contents, and KACC doenjang showed higher ammonia-type nitrogen (AMN) content. TBZA doenjang showed higher fibrinolytic and protease activity than other doenjang samples. Metabolites analyses by GC/MS showed that doenjang samples were separated from each other by partial least squares-discriminant analysis (PLS-DA) analysis. Seventeen major metabolites involved in the differences between samples were identified and they included organic acids, amino acids, sugars, fatty acids and alcohols. TBZA doenjang showed higher contents for most metabolites responsible for flavor and taste of fermented foods including doenjang. These results showed that *T. halophilus* could be useful as a starter for doenjang and can improve the product quality by accelerating the fermentation processes.**

Keywords: ***Tetragenococcus halophilus*****, doenjang, multiple starters, fermentation**

Introduction

Doenjang, a traditional Korean fermented soybean paste, is an important seasoning for Korean diets and serves as a source for fatty acids, organic acids, minerals, vitamins, and essential amino acids [1]. Traditionally, doenjang has been prepared from cooked soybean by natural fermentation, i.e. microorganisms in the surrounding environments carry out fermentation [2]. Fungi such as *Aspergillus* species (spp.) and bacteria such as *Bacillus* spp. and lactic acid bacteria (LAB) are the most important microorganisms for doenjang fermentation [3]. During fermentation, proteins and carbohydrates of soybean are hydrolyzed into peptides, amino acids, sugars, and organic acids, contributing to the unique taste and flavor of doenjang. Traditional doenjang fermentation method has some problems including long fermentation time, high salt contents, highly variable qualities, and danger of contamination by toxinogenic microorganisms such as mycotoxin producing fungi and toxin producing *Bacillus cereus* [4]. Starters have been used for the commercial doenjang production to avoid

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such problems. Currently used starters are mainly *Aspergillus oryzae* and *A. sojae*. Doenjang fermented with a single strain of *Aspergillus* spp. has a disadvantage of simple taste, contrasting to deep and complex taste of traditional doenjang [2, 4]. As an alternative, a *B. subtilis* strain isolated from traditional doenjang was used as the starter and the resulting doenjang showed good qualities [3].

We previously reported on doenjang fermented with multiple starters consisting of two *B. amyloliquefaciens* strains with high fibrinolytic activities and antimicrobial activities, *Pichia farinosa*, and *Rhizopus oryzae* [5, 6]. The resulting doenjang products were safe from contamination by *B. cereus* and fungi, and showed higher fibrinolytic activities than control doenjang prepared by natural fermentation. In this study, *Tetragenococcus halophilus* CY54 was included into multiple starters. The strain was isolated from myeolchi (anchovy) jeotgal and showed high protease activities [7]. *Tetragenococcus* spp. such as *T. halophilus* and *T. muriaticus* are halophilic LAB isolated from fermented foods with high salinities such as jeotgal, fish sauce, soy sauce, and doenjang [8–11]. *Tetragenococcus* spp. can grow up to 24% NaCl concentration and grow rapidly at 25–30℃. *Tetragenococcus* spp. have been tried as a starter for fish sauce and soy sauce fermentation to improve taste and flavor of products [12, 13].

In this study, doenjang was prepared by using multiple starters consisting of *T. halophilus*, *B. subtilis*, *Zygosaccharomyces rouxii* and *A. oryzae*, and the properties of doenjang were investigated.

Materials and Methods

Multiple starters

T. halophilus CY54 was cultivated in MRS broth (Difco Lab, USA) with 5% NaCl at 30℃. *B. subtilis* JS2 and *B. subtilis* KACC16750 were grown in Luria Bertani (LB, MB Cell, Korea) broth at 37℃ with shaking. *Z. rouxii* and *A. oryzae* were grown in yeast mold (YM, USA) medium at 25℃. Cells were recovered by centrifugation (12,000 ×*g* for 10 min at 4℃). Cell pellet was washed with sterile water 2 times, and resuspended in sterile water. Viable cells were counted by spreading serially diluted samples onto specific agar plates. For counting *A. oryzae* spores, mycelia on the YM agar plates were recovered using sterile chopsticks and resuspended in sterile water. After filteration with gauze, spores in the filtrate were counted using a hemocytometer (Paul Marienfeld GmbH & Co., Germany).

Preparation of doenjang samples

Soybeans (crop year, 2021, Hamyang, Gyeongnam, Korea) were washed, soaked in distilled water for 15 h at room temperature (RT), and autoclaved for 60 min at 121℃. Cooled soybeans were inoculated with each starter separately. Soybeans inoclulated with *Bacillus* spp. were incubated at 37℃, and soybeans inoculated with *T. halophilus* were incubated at 30℃. Soybeans inoculated with *Z. rouxii* or *A. oryzae* were incubated at 25℃. After 48 h of incubation, separately fermented soybeans were mixed together and the same amount of autoclaved soybeans were added. Salt (purified, 99% NaCl) and water were added. Each doenjang was 5 kg in

Table 1. Preparation of doenjang samples.

Doenjang sample	Starter soybean (kg)		Non-starter soybean (kg)	Salt (kg)	Water (kg)	Total (kg)
TBZA	*T. halophilus* CY54	0.375	1.500	0.460	1.540	5
	B. subtilis JS2	0.375				
	Z. rouxii	0.375				
	A. oryzae	0.375				
BZA	*B. subtilis* JS2	0.500	1.500	0.500	1.500	5
	Z. rouxii	0.500				
	A. oryzae	0.500				
KACC	*B. subtilis* KACC16750	1.500	1.500	0.500	1.500	5

*All doenjang samples were 5 kg with 10% NaCl (w/w).

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weight with 10% NaCl concentration (w/w). Three (TBZA, BZA and KACC) doenjang samples were prepared and the components of doenjang samples are shown in Table 1. Each starter was inoculated at 10^8 CFU (spore) per g of doenjang. Three doenjang samples were further fermented for 16 weeks at 25℃ inside closed plastic boxes. Samples were collected at intervals, and the samples were stored at deep freezer (-80℃) for later analyses.

Property changes of doenjang during fermentation

Viable counts, pH and titratable acidity (TA)

Ten gram of each doenjang sample was homogenized with 90 ml peptone water (0.1%, w/v) using a stomacher (Stomacher® 80, UK), and serially diluted with peptone water. MRS agar plates with 10% NaCl (w/v) and cycloheximide (50 μg/ml) were used for *T. halophilus* counting and LB agar plates containing cycloheximide (50 μg/ml) for bacilli counting. YM agar plates containing chloramphenicol (170 μg/ml) were used for yeast counting and potato dextrose agar (PDA, MBcell, Korea) plates with chloramphenicol (170 μg/ml) for fungi counting. Plates were incubated for 120 h at 30℃ for *T. halophilus* and yeast, and 24 h at 30℃ for bacilli, and 120 h at 25℃ for fungi.

pH was measured using a pH meter (Thermo Fisher, USA). TA was calculated by titrating supernatant with 0.1 N NaOH until pH 8.4. Measurements were repeated three times and the average values were used.

Moisture contents, salinity and crude protein contents

Moisture contents were measured by using an infrared moisture analyzer (MX-50, Japan). For salinity measurements, 10 g of each homogenate was mixed with 40 ml of distilled water, and shaken in a water bath for 1 h at 30℃. Supernatant was obtained by centrifugation and salinity was measured using a salt meter (PAL-SALT, Japan). Crude protein contents were determined by Dumas method (AOAC 990.03).

Amino-type nitrogen (ANN), Ammonia-type nitrogen (AMN), and volatile basic nitrogen (VBN) contents

Ten gram of each homogenate was mixed with 90 ml distilled water, and the mixture was shaken for 1 h at 30℃ in a water bath. After centrifugation, the obtained supernatants were used for measuring ANN, AMN and VBN contents as described previously [14].

Enzyme activities of doenjang samples

Freeze-dried doenjang (1 g) was resuspended in 50 ml distilled water and the mixture was shaken for 1 h at 30℃ in a water bath. Supernatant was obtained after centrifugation (12,000 ×*g*, 10 min) and filteration, and used as the sample for fibrinolytic activity measurement by fibrin plate method [5]. Samples for α-amylase and proteolytic activities measurements were prepared by the same way except 40 ml distilled water was used to dissolve 1 g freeze-dried doenjang. α-Amylase activity was measured by DNS method [15]. One unit (U) of α-amylase was defined as the amount of enzyme producing 1 μmol maltose per min under the corresponding conditions. Protease activities were measured at acidic, neutral and alkaline pH as described previously [8]. One unit of protease activity was defined as the amount of enzyme releasing 1 μmol of tyrosine per min.

Metabolomic analyses of doenjang by GC/MS

Freeze-dried doenjang was extracted with 80% methanol including phthalate as an internal standard. Extract (100 μl) was dried using a vacuum centrivap concentrator (Labconco, USA) at 40℃. Dried extract was resuspended with 70 μl methoxyamine hydrochloride in pyridine (20 mg/ml) and stood for 90 min at 70℃. Then 70 μl *N, O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was added and stood for 30 min at 37℃. The derivatized metabolites were analyzed by GC/MS using GC-2010 plus (Shimazu, Japan) equipped with a DB-5 capillary column (30 m × 0.25 mm id, 0.25 μm Agilent J&W, USA) as described previously [16]. The GC column effluent was analyzed by Shimadzu GCMS-TQ 8030 (Japan) with electron impact (EI) ionization mode at 70 eV. The ion source and interface temperature were 230℃ and 280℃, respectively. Effluents were monitored in the full scan mode in the range of 45–800 m/z with 0.3 sec of scan event time and 3333 u/sec of scan speed. Detector voltage was 0.1 kV and threshold was 100. Data obtained using GC/MS were aligned with retention time window of 0.1–0.05 min and normalized with the IS. Metabolites were identified using GC/MS database (NIST 11 and Wiley 9 mass spectral libraries) and retention indices (RI) calculated using *n*-alkanes [16].

Statistical analyses

GC/MS data were analyzed with multivariate statistical analysis using SIMCA-P+ version 16.0.1 (Umetrics, Sweden), and partial least squares discriminant analysis (PLS-DA) scores plot was used to visualize analysis results. The conformity of PLS-DA model was determined using goodness of fit (R^2X and R^2Y), predictability (Q^2Y), *p*-value, and cross-validation determined by the permutation test ($n = 200$). The statistical difference between the experimental data were analyzed using one-way analysis of variance (ANOVA) with Duncans test ($p < 0.05$) using SPSS 17.0 (SPSS Inc., USA).

Results and Discussion

Selection of multiple starters

T. halophilus CY54 grew optimally at 5–10% NaCl concentration, and could grow up to 20% NaCl. The safety of *T. halophilus* CY54 as a starter was confirmed [7]. *B. subtilis* JS2 was isolated from saeu (small shrimp) jeotgal and possessed strong fibrinolytic activity, and could grow at 20% NaCl (w/v) concentration [17]. *Z. rouxii* was selected since this organism was expected to contribute to flavor development of doenjang [18]. *A. oryzae* has strong proteolytic activities and has been used as a single starter for commercial doenjang production [19]. Each organism was tested for the compatibility with other organisms by cross-streaking and close inoculation on agar plates [20]. Co-cultivation was also tried by inoculating each strain at 10^8 CFU/ml in TSB with 5% NaCl (w/v). Co-inoculated culture was incubated for a week at 30℃, and viable cells were counted. Based on the results, multiple starters consisting of *T. halophilus* CY54, *B. subtilis* JS2, *Z. rouxii*, and *A. oryzae* were established.

Property changes of doenjang during fermentation

Viable counts, pH and titratable acidity (TA)

Initial viable counts of TBZA doenjang at 0 week (wk) were 2.13×10^8, 1.97×10^8, 3.13×10^8 and 1.31×10^8 CFU (spore)/g for *T. halophilus*, *B. subtilis*, *Z. rouxii,* and *A. oryzae*, respectively (Table 2). The highest counts of *T. halophilus*, *B. subtilis*, *Z. rouxii*, and *A. oryzae* were 2.92×10^8 (1 wk), 3.50×10^8 (10 wk), 5.07×10^8 (4 wk), and 6.30×10^8 CFU (spore)/g (8 wk), respectively. At 16 wk, viable counts of *T. halophilus*, *B. subtilis*, *Z. rouxii,* and *A. oryzae* were 4.33×10^7, 1.70×10^7, 1.40×10^7 and 3.43×10^7 CFU (spore)/g, respectively. The results showed that each starter grew differently from others. Net increases of each organism at the highest point were 1.37, 1.78, 1.62, and 4.81-fold for *T. halophilus*, *B. subtilis*, *Z. rouxii*, and *A. oryzae*, respectively. Increases were not great probably because initial inoculum size (1×10^8 CFU (spore)/g doenjang) was already high. *T. halophilus* showed the lowest increase and reached the highest count at 1 wk and then decreased slowly. *A. oryzae* showed the highest increase in the viable count. *A. oryzae* grew well at 10% NaCl content in preliminary tests.

Bacillus counts of BZA doenjang were maintained constantly during 16 wk, and reached the highest count (2.93×10^8 CFU/g) at 10 wk like TBZA doenjang. *Bacillus* count at 16 wk was 2.40×10^8 CFU/g, slightly higher than the initial count (1.90×10^8 CFU/g). *B. subtilis* JS2 can grow at 20% NaCl concentration, and thus expected to grow well in doenjang with 10% NaCl concentration.

Table 2. Viable counts of doenjang samples during fermentation (CFU (spore)/g).

		Fermentation period (weeks)											
		0	1	2	3	4	5	6	8	10	12	14	16
TBZA	LAB	2.13×10^8	2.92×10^8	2.03×10^8	2.01×10^8	2.17×10^8	1.30×10^8	1.37×10^8	1.11×10^8	1.07×10^8	6.30×10^7	7.57×10^7	4.33×10^7
	Bacillus	1.97×10^8	1.50×10^8	1.50×10^8	1.67×10^8	1.90×10^8	2.47×10^8	2.70×10^8	2.47×10^8	3.50×10^8	2.67×10^8	1.63×10^8	1.70×10^7
	Yeast	3.13×10^8	2.57×10^8	2.37×10^8	2.27×10^8	5.07×10^8	4.97×10^8	4.63×10^8	3.80×10^8	4.23×10^8	2.53×10^8	1.18×10^8	1.40×10^7
	Fungi	1.31×10^8	3.33×10^8	4.30×10^8	4.70×10^8	4.00×10^8	2.70×10^8	4.30×10^8	6.30×10^8	3.70×10^8	8.00×10^7	3.00×10^7	3.43×10^7
BZA	Bacillus	1.90×10^8	2.07×10^8	1.97×10^8	1.77×10^8	2.23×10^8	2.33×10^8	2.80×10^8	2.80×10^8	2.93×10^8	2.57×10^8	3.50×10^8	2.40×10^8
	Yeast	2.27×10^8	2.63×10^8	6.67×10^8	2.13×10^8	2.53×10^8	2.73×10^8	2.53×10^8	1.83×10^8	2.20×10^8	1.53×10^8	7.07×10^7	2.83×10^7
	Fungi	1.47×10^8	1.80×10^8	4.30×10^8	2.70×10^8	1.23×10^8	1.70×10^8	1.03×10^8	1.10×10^8	8.70×10^7	8.30×10^7	7.70×10^7	1.30×10^7
KACC	Bacillus	3.82×10^8	1.93×10^8	1.27×10^8	1.90×10^8	2.80×10^8	3.01×10^8	2.17×10^8	2.60×10^8	1.10×10^8	1.37×10^8	1.33×10^8	1.50×10^8

*: Values represent the mean ± standard deviation.

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Bacillus counts of BZA doenjang were higher than those of TBZA doenjang during fermentation except at 5, 10, and 12 wk. Although it was confirmed that *T. halophilus* CY54 did not inhibit *B. subtilis* JS2 by compatability tests, some inhibition by *T. halophilus* CY54 was possible in TBZA doenjang. The counts of *Z. rouxii* and *A. oryzae* reached the highest points at 2 wk, and then gradually decreased. *Z. rouxii* and *A. oryzae* showed higher viable counts increases (2.94 and 2.93 fold) than *B. subtilis* JS2 (1.84 fold). *Z. rouxii* maintained the initial count until 12 wk and *A. oryzae* until 8 wk.

Initial *B. subtilis* KACC16750 count in KACC doenjang was 3.82×10^8 CFU/g and this was the highest number throughout fermentation. Viable count decreased slowly and the final count was 1.50×10^8 CFU/g. *B. subtilis* KACC16750 did not grow at 10% NaCl but remained viable. *Bacillus* spp. such as *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* are believed to play some important roles during doenjang fermentation and many grow well at 10% and higher NaCl concentration [21].

Initial pH values were 4.87 ± 0.01, 5.63 ± 0.04, and 5.70 ± 0.01 for TBZA, BZA, and KACC doenjang, respectively (Fig. 1). TBZA doenjang showed the lowest pH and the lower pH of TBZA doenjang must be due to *T. halophilus* CY54, a homolactic fermenter producing lactic acid. pH decreased rapidly during the first 3 wk and then remained constant. The final pH values were 4.66 ± 0.00, 4.83 ± 0.01 and 4.87 ± 0.01 for TBZA, BZA, and KACC doenjang, respectively, slightly lower than the initial values. TA changed similarily with pH values but in an opposite direction (Fig. 1). Initial TA values were 0.66 ± 0.03, 0.41 ± 0.00 and 0.39 ± 0.00 for TBZA, BZA and KACC doenjang, respectively, and increased gradually during fermentation. Decrease in pH and increase in TA are common phenomena caused by acid production by lactic acid bacteria and other microorganisms [22].

Moisture contents, salinity and crude protein contents

Moisture contents immediately after doenjang preparation were 55.62 ± 0.34%, 53.89 ± 0.64% and 56.19 ± 0.75% for TBZA, BZA and KACC doenjang, respectively. No significant changes were observed during 16 wk of fermentation because doenjang samples were stored in a closed plastic box in an incubator, which prevented evaporation of moisture. Moisture contents of doenjang samples were similar with those of traditional doenjang (49.8–58.9%) [23]. Initial salinities were 10.03 ± 0.06%, 10.23 ± 0.03%, and 10.45 ± 0.13% for TBZA, BZA and KACC doenjang, respectively, and no significant changes observed during fermentation.

Immediately after preparation, crude protein contents were 18.99, 18.18, and 18.75% for TBZA, BZA, and KACC doenjang, respectively. Crude protein contents decreased slightly at 16 wk compared with the initial contents, and this was caused by protein hydrolysis during fermentation.

Amino-type nitrogen (ANN), Ammonia-type nitrogen (AMN), and volatile basic nitrogen (VBN) contents

ANN contents of doenjang samples increased during fermentation (Fig. 2A). ANN contents of fermented foods are related with the degree of protein hydrolysis of raw materials. Proteins are degraded into peptides, amino acids, amines, and ammonia by proteolytic enzymes from microorganisms or raw materials during fermenta-

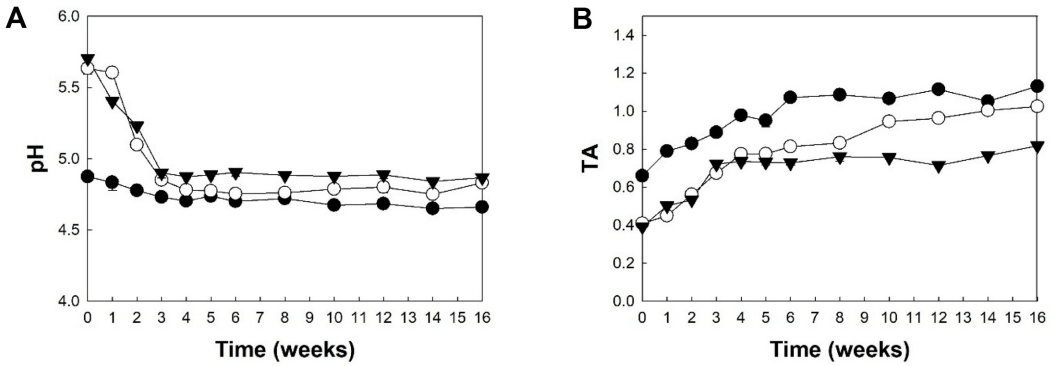

Fig. 1. pH (A) and TA (B) of doenjang samples during fermentation. ●, TBZA doenjang; ○, BZA doenjang; ▼, KACC doenjang.

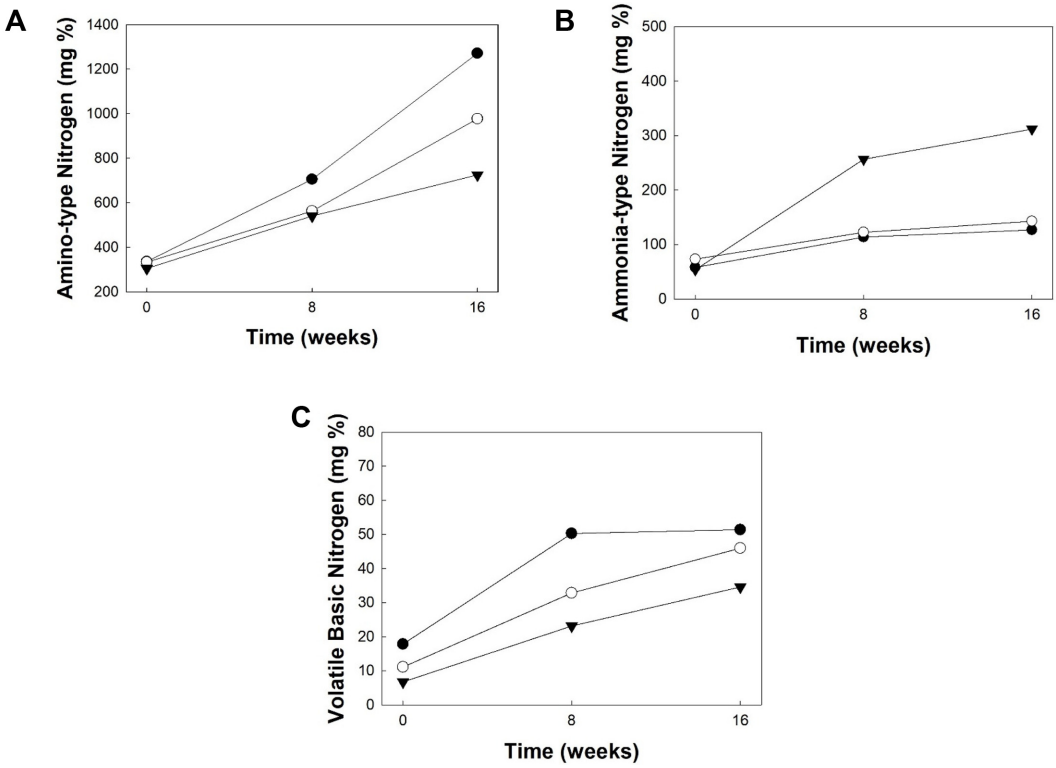

Fig. 2. ANN (A), AMN (B) and VBN (C) contents of doenjang samples. ●, TBZA doenjang; ○, BZA doenjang; ▼, KACC doenjang.

tion. These compounds confer doenjang unique physical properties, flavor, and aroma. ANN content is used as an indicator showing the degree of ripening of doenjang. Rapid increases in ANN were observed from all doenjang samples. TBZA doenjang showed higher increase than BZA and KACC doenjang, and this was expected because *T. halophilus* CY54 possessed strong proteolytic activities [7]. BZA doenjang showed higher ANN than KACC doenjang. It can be concluded that *T. halophilus* CY54 could be used to accelerate doenjang fermentation.

Excessive decomposition of proteins leads to production of AMN compounds with strong volatility, causing unpleasant flavor. But AMN content is also used as an index for fermented foods. KACC doenjang showed higher AMN contents than TBZA and BZA doenjang, both showing similar AMN contents (Fig. 2B). The results indicated that multiple starters (TBZA and BZA) were better than single starter (KACC) in terms of flavor of the final doenjang products. VBN contents increased steadily during fermentation period, and TBZA doenjang showed the highest values (Fig. 2C). VBNs are small nitrogen compounds with volatility, such as various basic amines including ammonia and trimethylamine [24]. Excessive VBN in fermented foods is often related with inferior food quality because of volatile off-flavors. The higher VBN content of TBZA doenjang might be an indication of its higher proteolytic activity. As the fermentation progressed, contents of crude protein were reduced but contents of peptides, amino acids, amines, and ammonia were increased, resulting in increases in ANN, AMN, and VBN. Above results indicated that *T. halophilus* CY54 contributed to the doenjang fermentation.

Enzyme activities of doenjang during fermentation

Fibrinolytic activities of TBZA (200.45 mU/μl) and BZA (166.21 mU/μl) doenjang were much higher than that of KACC doenjang (13.86 mU/μl) (Fig. 3A). This was due to *B. subtilis* JS2, a strain with strong fibrinolytic activity [17]. Fibrinolytic activity of TBZA doenjang was higher than that of BZA doenjang, indicating that *B. subtilis* JS2 was not significantly inhibited by *T. halophilus* CY54. Fibrinolytic activity of KACC doenjang was insignificant, indicating the strain did not produce

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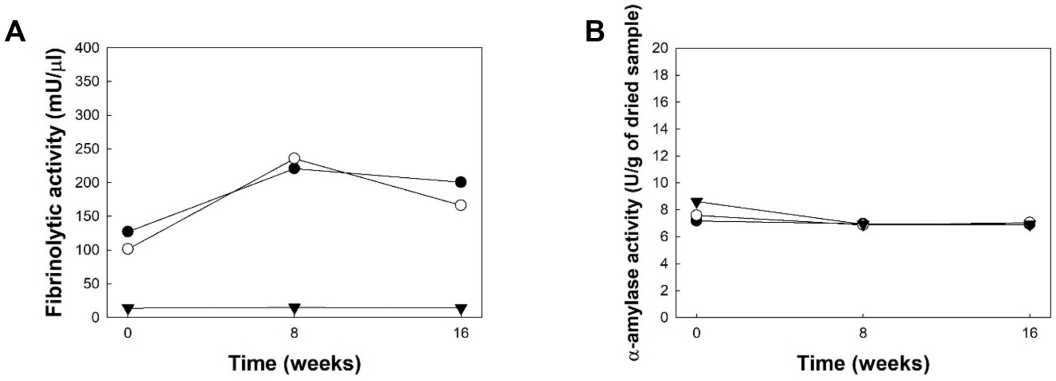

Fig. 3. Fibrinolytic activities (A) and α-amylase activities (B) of doenjang samples. ●, TBZA doenjang; ○, BZA doenjang; ▼, KACC doenjang.

fibrinolytic enzyme. α-Amylase activity is commonly observed among most *Bacillus* spp., and all doenjang showed similar values (6.91–7.04 U/g) (Fig. 3B).

Protease activities of doenjang samples were measured at acidic, neutral, and basic pH. All three doenjang showed similar activities. However, activity of TBZA doenjang gradually increased as fermentation progressed compared to BZA and KACC doenjang, and showed the highest activity at 16 wk. TBZA doenjang was expected to show higher protease activity because *T. halophilus* CY54 showed high protease activity.

Metabolomic analyses of doenjang by GC/MS

Differences in metabolites between samples were visualized by PLS-DA score plot (Fig. 4). The goodness of fit ($R^2X = 0.510$; $R^2Y = 0.962$), predictability ($Q^2 = 0.908$), p-value (4.21×10^{-6}), and permutation test of the PLS-DA model indicated that the model was statistically acceptable. In PLS-DA score plot, BZA sample was clearly separated from other samples by t(1). KACC and TBZA doenjang were separated from each other by t(2). The variable importance in the projection (VIP) and p-value of the metabolites were analyzed to identify the

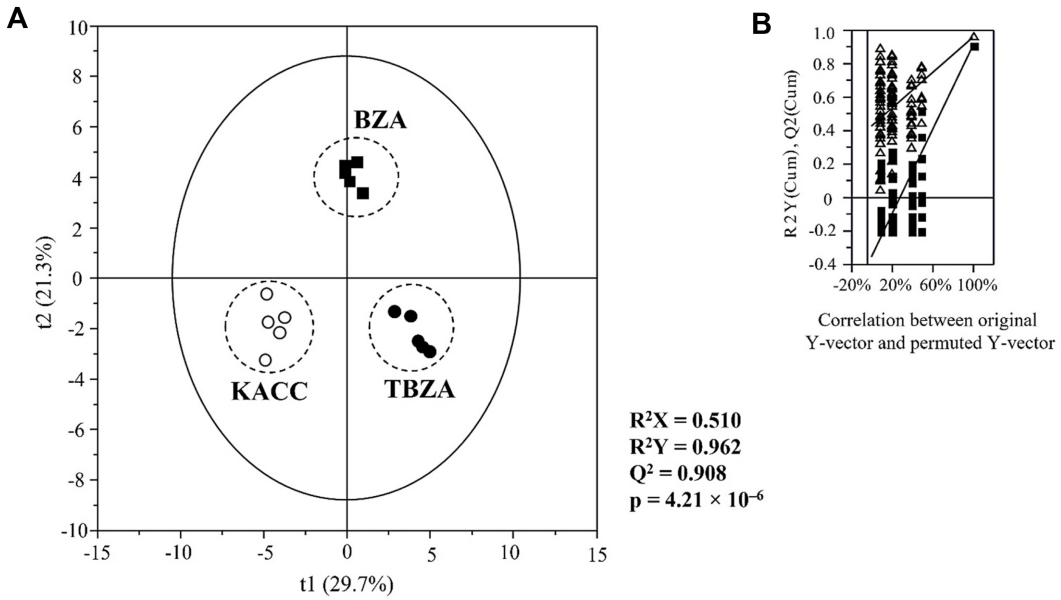

Fig. 4. PLS-DA score plots of doenjang samples. GC/MS analyses of doenjang samples at 16 wk were used. The qualities of the PLS-DA score plots for the metabolites were evaluated by R^2X, R^2Y, Q^2, and p-values (A) and validated by the permutation test (n = 200) (B).

Table 3. Major metabolites contributing to the separation among samples based on the PLS-DA scores plots of the data analyzed by GC/MS.

NO	Metabolites	RT[a]	RI[b]	VIP[c]	*p*-value[d]
1	Propylene glycol	5.67	1025	1.096	1.24×10^{-2}
2	2,3-Butanediol	5.82	1035	1.358	0.01×10^{-2}
3	Lactic acid	6.11	1052	1.061	1.12×10^{-2}
4	Oxalic acid	7.46	1135	0.960	2.42×10^{-2}
5	Valine	8.67	1210	0.958	1.20×10^{-2}
6	Leucine	9.54	1266	1.172	0.51×10^{-2}
7	Proline	9.95	1292	1.217	0.01×10^{-2}
8	Threonine	11.21	1377	1.193	0.01×10^{-2}
9	Aspartic acid	13.05	1512	1.076	0.18×10^{-2}
10	Glutamic acid	14.33	1612	1.160	0.04×10^{-2}
11	Phenylalanine	14.45	1622	0.948	1.45×10^{-2}
12	Fructose	14.82	1652	1.318	0.01×10^{-2}
13	Arabitol	15.51	1709	1.413	0.01×10^{-2}
14	Citric acid	16.66	1809	1.407	0.01×10^{-2}
15	Mannitol	17.87	1920	1.483	0.01×10^{-2}
16	Tyrosine	18.01	1932	1.141	0.13×10^{-2}
17	Palmitic acid	19.13	2041	1.044	0.93×10^{-2}

[a] RT is retention time.
[b] RI is retention indices calculated with *n*-Alkanes.
[c] Variable importance in the projection (VIP) values were determined by PLS-DA.
[d] *p*-Values were analyzed by ANOVA with Duncan's test.

metabolites contributing to the differences (Table 3). Seventeen metabolites that have higher VIP (>0.9) and lower *p*-value (<0.05) were identified as the major metabolites contributing to the differences (Table 3), and they included sugars (fructose, arabitol, and mannitol), amino acids (valine, leucine, proline, threonine, aspartic acid, glutamic acid, phenylalanine, and tyrosine), organic acids (lactic acid, oxalic acid, and citric acid), and fatty acid (palmitic acid). These metabolites are important for the taste and flavor of fermented foods including doenjang and their amounts are subjected to changes by the fermentation conditions [25].

Normalized intensities of all identified metabolites were compared and the metabolic pathways were proposed (Fig. 5). Levels of the primary metabolites varied markedly among doenjang samples. TBZA doenjang contained relatively higher contents in organic acids except citric acid. Lactic acid was produced a lot by *T. halophilus* CY54 in TBZA doenjang.

Amino acids are a good source of nutrients and also important for flavor of foods. Twelve amino acids were found to be significantly involved in doenjang fermentation (results not shown). Alanine and threonine are associated with sweet taste, and aspartic acid, glutamic acid and lysine are relevant to umami taste. Isoleucine, leucine, phenylalanine, proline and valine are responsible for bitter taste of doenjang. Most amino acids were significantly abundant in TBZA doenjang, and KACC doenjang showed the lowest contents in all amino acids (results not shown). This was related with the fact that TBZA doenjang showed higher protease activity. Fructose showed a significant value only in TBZA doenjang and the contents of mannitol, myo-inositol and arabitol were higher in TBZA and BZA doenjang than KACC doenjang (results not shown). Metabolite analyses indicated that TBZA doenjang was better than other doenjang samples in terms of flavor and taste.

The potential of *T. halophilus* CY54 as a member of multiple starters for doenjang fermentation was investigated. TBZA doenjang showed lower pH, higher TA values than other doenjang samples. ANN content, fibrinolytic activity and protease activity of TBZA doen-

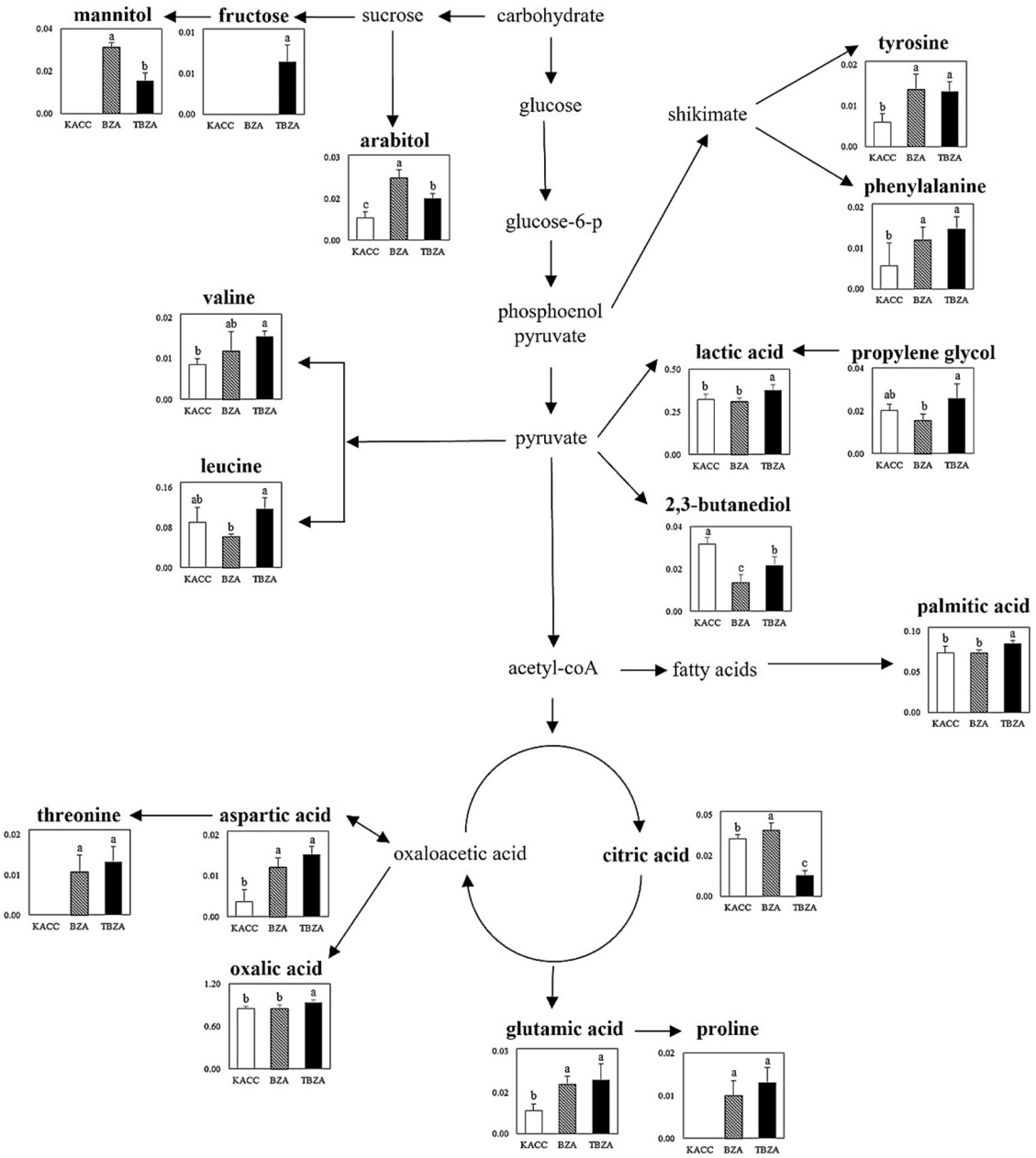

Fig. 5. Proposed metabolic pathways for doenjang fermentation. Metabolic pathways associated with energy production and the relative quantitative analyses of identified metabolites were used. The metabolic pathway was adopted from the KEGG database (https://www.kegg.jp/). The horizontal axis of the bars shows the normalized intensity, and different letters on the bars represents significant differences at $p < 0.05$.

jang were higher than those of other doenjang samples, indicating positive roles of *T. halophilus* for doenjang fermentation. TBZA doenjang contained comparatively higher contents of nutritional compounds such as amino acids and organic acids. It can be concluded that TBZA doenjang was the best in flavor and taste. The results suggest that *Tetragenococcus* strains can serve as a starter for fermented foods with high salinities such as doenjang and jeotgals, improving the qualities of products. Each starter organism was reasonably compatible with other starters in TBZA and BZA doenjang samples, showing the potential of multiple starters for doenjang

fermentation. Further studies are necessary on the roles of each starter for doenjang fermentation together with development of economical methods for inoculating multiple starters into soybean.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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