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ARTICLE

Safety and biotechnological properties of *Enterococcus faecalis* and *Enterococcus faecium* isolates from *Meju*

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Abstract We assessed the safety and biotechnological properties of 17 *Enterococcus faecalis* and 18 *Enterococcus faecium* strains isolated from *Meju* to select starter candidates for quality *Meju* production. Minimum inhibitory concentration assays showed that all strains were susceptible to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin G, and tetracycline, as judged by the breakpoint values defined at the species level by the European Committee on Antimicrobial Susceptibility Testing, whereas two *E. faecalis* strains were resistant to vancomycin, which could be ascribed to acquired resistance. None of the strains exhibited α- or β-hemolytic activities or biofilm formation. Both species showed similar levels of biogenic amine production, and noticeable amounts of tyramine (3397.4 ± 172.4 ppm) were produced by all strains in the presence of precursors (histidine, lysine, ornithine, and tyrosine). All strains exhibited protease, lipase, and acid production, with decreased activity observed with increasing NaCl concentrations. This study confirmed the necessity of antibiotic resistance screening of *Enterococcus* species for food production, with the identification of vancomycin-resistant strains from *Meju*.

Keywords *Doenjang*, Korean fermented soybean paste · *Enterococcus faecalis* · *Enterococcus faecium* · *Meju*

Introduction

Doenjang, a traditional Korean fermented soybean paste, literally means thick sauce in Korean and is used as a sauce for vegetables, fish, and meats and as an ingredient in soup for additional protein and flavor. This soybean product is made by the mixing and ripening of *Meju* with a high salt brine. *Meju* is a naturally fermented soybean product prepared by soaking, steaming, crushing, and molding the soybean into blocks and allowing it to ripen for 1–2 months. Naturally occurring fungi, yeast, and bacteria are involved in the fermentation of both *Meju* and *Doenjang*. Early microbiological studies of fermented soybean foods from Korea suggested that *Bacillus* species and *Aspergillus oryzae* played major roles in the fermentation, because they were frequently isolated and exhibited high amylase and protease activities (Kim et al. 2006, 2013; Kim and Kwon 2014). However, recent studies performed using culture-independent methods have reported the involvement of a wider variety of microorganisms, including lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS). Bacilli were the predominant group of bacteria, and enterococci were the representative LAB found in both *Meju* and *Doenjang* (Nam et al. 2012; Jung et al. 2014).

Recently, we evaluated the bacterial diversity of these products using culture-dependent methods to understand the living bacterial community migration from *Meju* to *Doenjang* (Jeong et al. 2014b). *Enterococcus faecalis* and *Enterococcus faecium* were the predominant species in the *Meju* fermentation, together with bacilli and CNS. Both

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species of *Enterococcus* can grow in medium containing up to 7 % (w/v) NaCl and exhibit protease and lipase activities, as well as acid production. Thus, these species can enhance the aromatic value when applied as starter cultures for the mass production of quality *Meju*.

The safety of *Enterococcus* species should be thoroughly examined prior to their use in food fermentation because of the potential for acquisition of virulence factors. Strains carrying multiple antibiotic resistance determinants, along with virulence factors including adhesions, invasions, and hemolysin, have been reported (Fifadara et al. 2003; Martin et al. 2005; Domann et al. 2007). Enterococci have also been identified as nosocomial pathogens that can cause bacteremia, endocarditis, and other infections (Franz et al. 1999; Zheng et al. 2009). Therefore, numerous studies have attempted to identify strains that can safely be used as starters or probiotics (Fifadara et al. 2003; Bhardwaj et al. 2008; Franz et al. 2011). However, to the best of our knowledge, no safety assessment of *E. faecalis* and *E. faecium* isolates from *Meju* has been performed. In this study, we assessed the safety and biotechnological properties of *E. faecalis* and *E. faecium* strains isolated from *Meju* to select possible starter strains for *Meju* manufacture, and to ascertain the general safety of Korean isolates.

Materials and methods

Bacterial strains and culture conditions

Seventeen *E. faecalis* and 18 *E. faecium* strains previously isolated from *Meju* (Jeong et al. 2014b) were used in the current study. Strains were cultured in de Man–Rogosa–Sharpe medium (MRS; Difco, USA) at 30 °C for 24 h.

Determination of minimum inhibitory concentrations (MICs)

Antibiotic MICs were determined by the broth micro-dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (2007). Eight antibiotics from among those frequently used to test antibiotic sensitivity of LAB were selected (ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, and vancomycin) (Pinheiro et al. 2004; Perez-Pulido et al. 2006; Perin et al. 2014). A twofold serial dilution was prepared for each antibiotic in deionized water, and the final concentrations in each well of the microplate ranged from 1 to 512 mg/L. *Enterococcus* strains were cultured twice in MRS broth and matched to a McFarland 0.5 turbidity standard (bioMerieux, France). The cultured strains were further diluted (1:100) into cation-adjusted Mueller–Hinton broth (Oxoid, UK) containing 2 % (v/v) horse blood (KisanBio, Korea) to achieve the desired inoculum concentration. The final inoculum density in each well was 5×10^5 colony forming units/mL. Microplates were then incubated at 30 °C for 24 h. The MIC of each antibiotic was recorded as the lowest concentration at which no turbidity was observed in the wells. Resistance to a particular antibiotic was defined as when the MIC (mg/L) value of a tested antibiotic was higher than the recommended breakpoint value defined at the species level by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org). All of the experiments were conducted at least three times on separate days.

Hemolytic activity

Tryptic soy agar (TSA, Difco) supplemented with 5 % (v/v) rabbit blood (MB Cell, Korea) and 5 % (v/v) sheep blood (BBL Microbiology Systems, USA) was used for α- and β-hemolytic activity tests, respectively. α-Hemolytic activity was determined by incubation at 30 °C for 24 h, while β-hemolytic activity was determined by cold shock at 4 °C for 24 h after incubation at 30 °C for 24 h, as described previously (Jeong et al. 2014a). Hemolytic activities were determined by the formation of clear lytic zones around colonies on each blood-containing TSA plate. *Staphylococcus aureus* USA300-P23 was used as a positive control for hemolytic analyses (Jeong et al. 2011). Experiments were conducted independently at least three times.

Biofilm formation assay

An overnight culture in tryptic soy broth (TSB, Difco) was diluted 200-fold with fresh TSB containing 0.5 % (w/v) glucose. A 200-μL aliquot of culture was then added to each well of a 96-well microtiter plate and incubated for 24 h at 37 °C without shaking. After the medium was discarded, the plate was dried, and the cells were stained with 0.1 % safranin (Heilmann et al. 1996). A positive safranin staining result indicated a biofilm formation. *Staphylococcus equorum* DSM 20674^T was used as a positive control for biofilm formation analysis (Jeong et al. 2013). Experiments were conducted independently at least three times.

Analysis of biogenic amine production

Cells from an overnight culture of each strain were normalized by measuring optical density at 600 nm, and then 1:100 dilutions of each normalized culture were inoculated into MRS broth containing four biogenic amine precursors. The precursors, L-tyrosine disodium salt hydrate, L-histidine monohydrochloride monohydrate, L-lysine monohydrochloride, and

L-ornithine monohydrochloride (pH 5.8), were added to a final concentration of 0.25 % (w/v), and pyridoxal-HCl was added to a final concentration of 0.0005 % (w/v) (Sigma, USA) (Bover-Cid and Holzapfel 1999). Cultures were incubated for 2 days at 30 °C under semi-aerobic conditions, and then 2-mL aliquots of the culture broths were dansylated with dansyl chloride according to a previously described method (Hwang et al. 1997) with minor modifications. The dansyl derivatives of any biogenic amines were dissolved in 5 mL of acetonitrile, and 10-μL aliquots were used for high-performance liquid chromatography (HPLC). Calibration curves for quantification were constructed using each authentic compound.

The biogenic amines produced by the strains were determined using an Agilent Technologies HPLC 1200 series system (USA) monitored by UV detector at 254 nm, and a Nova-Pak C18 column (4 μm, 150 × 4.6 mm) was used for chromatographic separation. The gradient elution program began with 50:50 (v/v) acetonitrile:0.1 M ammonium acetate at a flow rate of 1 mL/min, followed by a linear increase to 90:10 acetonitrile:0.1 M ammonium acetate for 19 min, and then decreased to 50:50 over the final 2 min. Experiments were conducted independently at least three times.

Amplification of the tyrosine decarboxylase gene

The genomic DNA of each of the *Enterococcus* strains was extracted using a DNeasy Tissue Kit (Qiagen, Germany). Polymerase chain reaction (PCR) amplification of the tyramine decarboxylase gene was performed using the specific primer set: forward, 5′-GAYATNATNGGNATNGGNYTN GAYCARG-3′ and reverse, 5′-CCRTARTCNGGNATAGC RAARTCNGTRTG-3′, as described previously (de Las Rivas et al. 2005), using a T-3000 thermocycler (Biometra, Germany). The PCR mixture consisted of the template DNA, 0.5 μM of each primer, 1.25 units of Inclone *Taq* polymerase (Inclone Biotech, Korea), 100 mM dNTPs, and 2.5 mM $MgCl_2$. Samples were preheated for 5 min at 95 °C and then amplified using 30 cycles of 1 min at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. The amplified PCR products were sequenced using a custom service provided by GenoTech (Korea). The web-hosted BLAST program was used to identify sequence homologies of the amplified fragments with known gene sequences from the GenBank database.

Determination of salt tolerance, protease and lipase activities, and acid production

Salt tolerance of the *Enterococcus* strains was determined by examining growth on MRS agar supplemented with NaCl at concentrations up to 10 % (w/v). Growth on 0, 5, and 10 % NaCl was observed after 1, 2, and 3 days of incubation, respectively. Protease activity was determined on TSA containing 2 % skim milk (w/v), and lipase activity was tested on tributyrin agar (Sigma) containing 1 % tributyrin (v/v). The tributyrin-supplemented medium was emulsified by sonication prior to autoclaving. Acid production was determined on TSA supplemented with 1 % glucose (w/v) and 0.7 % $CaCO_3$ (w/v). Colonies cultured on TSA were transferred to each substrate-supplemented agar medium and incubated at 30 °C for 48 h. The size of the zone of clearing around the colony was used as the indicator of enzyme activity. The effect of NaCl on each activity was determined by the addition of NaCl to each medium, up to a concentration of 6 % (w/v).

Results

Prevalence of antibiotic resistance and virulence factors

The MICs of the tested antibiotics for the 35 *Enterococcus* strains are summarized in Table 1. All strains were susceptible to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, penicillin G, and tetracycline. Twenty-two strains were resistant to erythromycin (≥8 mg/L) according to the breakpoint values for the particular species (*E. faecalis*, 4 mg/L; *E. faecium*, 4 mg/L). Two *E. faecalis* strains, 7AME16 and 7AME17, exhibited growth in the presence of 512 mg/L vancomycin.

No α- or β-hemolysis was observed for any of the strains on the blood-supplemented agar, and none of the strains formed a biofilm (data not shown).

In vitro biogenic amine production by *Enterococcus* strains

The production of four kinds of biogenic amines, which are the most prevalent biogenic amines found in fermented soybean products in Korea (Kim et al. 2001; Cho et al. 2006; Shukla et al. 2010), by the 35 *Enterococcus* strains is shown in Fig. 1. The patterns of biogenic amine production were similar for *E. faecalis* and *E. faecium*, and did not vary significantly among strains. None of the strains produced histamine, and cadaverine and putrescine levels were 14.3 ± 1.7 and 64.3 ± 4.2 ppm, respectively. However, all strains produced tyramine at levels greater than 3000 ppm (3397.4 ± 172.4 ppm).

Tyrosine decarboxylase is involved in the production of tyramine from tyrosine, and the gene coding for this protein was identified in the genomes of both *E. faecalis* strain 62 (GenBank accession no. NC_017312.1) and *E. faecium* strain Aus0004 (GenBank accession no. NC_017022.1). The tyrosine decarboxylase gene was amplified from all strains

Table 1 MICs of eight antibiotics for *Enterococcus* isolates from *Meju*

Antibiotic	Species	No. of isolates	MIC (μg/mL)										Breakpoint (μg/mL)[a]
			1	2	4	8	16	32	64	128	256	512	
Ampicillin	*E. faecalis*	17	0	17	0	0	0	0	0	0	0	0	4
	E. faecium	18	0	4	14	0	0	0	0	0	0	0	4
Chloramphenicol	*E. faecalis*	17	0	0	0	1	16	0	0	0	0	0	32
	E. faecium	18	0	0	0	9	9	0	0	0	0	0	32
Ciprofloxacin	*E. faecalis*	17	0	10	7	0	0	0	0	0	0	0	4
	E. faecium	18	1	13	4	0	0	0	0	0	0	0	4
Erythromycin	*E. faecalis*	17	0	1	5	11	0	0	0	0	0	0	4
	E. faecium	18	0	0	7	11	0	0	0	0	0	0	4
Gentamicin	*E. faecalis*	17	0	0	0	7	10	0	0	0	0	0	32
	E. faecium	18	0	0	9	5	4	0	0	0	0	0	32
Penicillin G	*E. faecalis*	17	0	0	17	0	0	0	0	0	0	0	16
	E. faecium	18	0	0	14	4	0	0	0	0	0	0	16
Tetracycline	*E. faecalis*	17	17	0	0	0	0	0	0	0	0	0	4
	E. faecium	18	18	0	0	0	0	0	0	0	0	0	4
Vancomycin	*E. faecalis*	17	1	3	11	0	0	0	0	0	0	2	4
	E. faecium	18	11	2	5	0	0	0	0	0	0	0	4

[a] The recommended breakpoint value defined at the species level by the EUCAST: http://mic.eucast.org

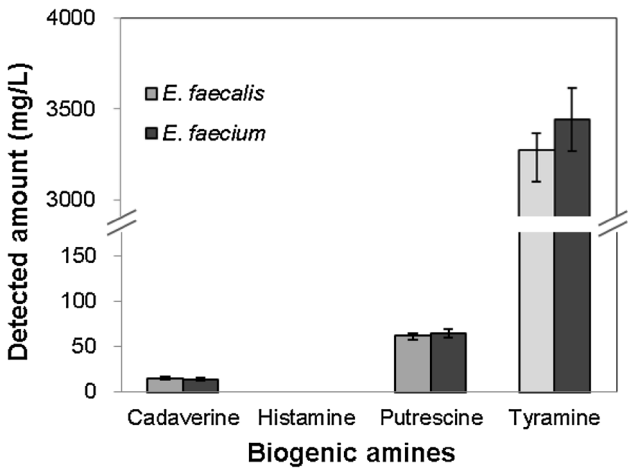

Fig. 1 Biogenic amine production by *Enterococcus* strains in medium supplemented with precursors

examined in the current study (data not shown), which agrees with the high levels of tyramine produced by all strains.

Biotechnological properties of candidate starter strains

Starter cultures have not previously been applied in the manufacturing of *Meju*; thus the required biotechnological parameters of starter cultures for *Meju* fermentation have not been determined. Considering the nature of soybean, which has a high protein and lipid content as well as fermentable sugars, the protease and lipase activities, along with the acid production of each strain, were measured by the degradation of substrates supplemented in agar medium (Table 2). To determine whether *Enterococcus* species can migrate from *Meju* to *Doenjang* during the *Doenjang* manufacturing process, we tested the growth of *E. faecalis* and *E. faecium* strains in the presence of 10 % NaCl. There was very little variation among the strains and all grew in media containing NaCl up to a concentration of 7 % (data not shown), which is not enough to survive during *Doenjang* fermentation. Generally, the protease and lipase activities of strains within a species were not significantly different, but variation among strains was detected as the NaCl concentration increased. *E. faecalis* strains exhibited higher protease activities than *E. facium* strains, and *E. faecalis* enzyme activity was maintained in the medium containing up to 6 % NaCl. Two *E. faecalis* strains and one *E. faecium* strain showed considerable lipase activity at 4 % NaCl. All the strains produced acid up to 6 % NaCl, but the growth rate was significantly decreased as NaCl concentrations increased.

Discussion

The fermentative LAB are categorized as "generally recognized as safe (GRAS)" microorganisms because of a long history of safety in the fermentation of natural

Table 2 Properties of *Enterococcus* isolates and effects of NaCl on activity levels

Species	Strain	Growth			Protease			Lipase			Acid production		
		0 %	5 %	10 %	0 %	3 %	6 %	0 %	2 %	4 %	0 %	3 %	6 %
E. faecalis	7AME9	++	++	N	++	++	+	+	+	−	++	++	++
	7AME11	++	++	N	++	++	+	+	+	+	++	++	+
	7AME12	++	++	N	++	++	++	+	W	W	++	++	+
	7AME13	++	++	N	++	++	+	+	−	−	++	++	+
	7AME14	++	++	N	++	++	+	+	W	+	++	++	+
	7AME19	++	++	N	++	++	+	+	+	−	++	++	+
	0BML21	++	++	N	++	++	++	+	+	W	++	++	+
	0AME11	++	++	N	++	++	++	+	+	−	++	++	+
	0AME14	++	++	N	++	++	++	+	+	+	++	++	+
	0AME15	++	++	N	++	++	++	+	+	−	++	++	+
	0AME16	++	++	N	++	++	++	+	+	−	++	++	+
	0AME17	++	++	N	++	++	++	+	+	−	++	++	+
	0AME18	++	++	N	++	++	++	+	+	−	++	++	+
	0AME22	++	++	N	++	++	+	+	+	−	++	++	+
	7AME16	++	++	N	++	++	+	+	+	W	++	++	+
	7AME17	++	++	N	++	++	+	+	+	W	++	++	+
	7AME18	++	++	N	++	++	+	+	+	W	++	++	+
E. faecium	0AME12	++	++	N	+	+	−	+	W	−	++	++	+
	0AME13	++	++	N	+	+	−	+	W	−	++	++	+
	0AME19	++	++	N	+	+	−	+	+	−	++	++	+
	0AME20	++	++	N	+	+	−	+	+	+	++	++	+
	0AME23	++	++	N	+	+	−	+	−	−	++	++	++
	0AME24	++	++	N	+	+	−	+	W	−	++	++	+
	0AME25	++	++	N	+	+	−	+	−	−	++	++	+
	0BME4	++	++	N	+	+	−	+	W	−	++	++	+
	0BME5	++	++	N	+	+	−	+	W	W	++	++	+
	0BME6	++	++	N	+	+	−	+	W	−	++	++	+
	0BME7	++	++	N	+	+	−	+	W	−	++	++	+
	0BML19	++	++	N	+	+	−	+	W	−	++	++	+
	0BML20	++	++	N	+	+	−	+	W	−	++	++	+
	0BML22	++	++	N	+	+	−	+	−	−	++	++	+
	0BML23	++	++	N	+	+	−	+	+	−	++	++	+
	0BME1	++	++	N	++	+	−	+	+	W	++	++	+
	0BME2	++	++	N	++	+	−	+	+	W	++	++	+
	0BME3	++	++	N	++	+	−	+	+	−	++	++	++

+, positive activity; W, weak activity; −, negative activity; N, non-growth

products and probiotics with health benefits. However, there has been a recent increase in the number of reports of their association with serious infections, including bacteremia, endocarditis, and localized abscesses (Aguirre and Collins 1993; Adams 1999; Franz et al. 2011). Therefore, these organisms are no longer automatically considered safe and are subject to in-depth consideration of potential safety issues. The genus *Enterococcus* is the most controversial group of LAB. *Enterococcus* species are principally an essential part of the microflora of humans and animals and are present in many different habitats, including soil, surface water, and plants, as well as in fermented foods. They have contributed significantly to the food industry, playing positive roles such as aroma enhancement in cheese and oleuropein breakdown in olives (Gardiner et al. 1999; Santos et al. 2012). In particular, *E. faecium* and *E. faecalis* have been used as probiotics for human consumption, as well as veterinary feed supplements. At the same time, several reports have shown that enterococci are the most common nosocomial pathogens and have been

implicated in cases of endocarditis, bacteremia, and infections of the urinary tract (Adams 1999; Franz et al. 2011).

Enterococcus species show very few intrinsic virulence traits; however, they can easily acquire virulence factors, especially antibiotic resistance, when they are exposed to antibiotic pressure (Patel et al. 2013). Notably, vancomycin-resistant enterococci have steadily increased in prevalence over the past two decades (Endtz et al. 1999). The National Healthcare Safety Network reported that 99.5 and 91.9 % of clinical *E. faecium* and *E. faecalis* strains, respectively, showed resistance to vancomycin (Hidron et al. 2008). Despite this, antibiotic resistance is far less common among enterococci of food origin. All enterococci from fermented capers were sensitive to vancomycin and showed low levels of resistance against other antibiotics (Perez-Pulido et al. 2006). In the current study, only two *E. faecalis* strains, 7AME16 and 7AME17, were resistant to vancomycin. The MICs of these two strains (512 mg/L) were 128 times higher than the suggested EUCAST breakpoint values for *E. faecalis* and *E. faecium* (both 4 mg/L), with resistance likely to be the result of acquisition of a vancomycin resistance gene through horizontal gene transfer.

Interestingly, 62.8 % (22 of 35) of strains examined in the current study exhibited erythromycin resistance, and the MICs were twofold higher than the suggested EUCAST breakpoint (4 mg/L). The MICs are unlikely to be the result of resistance acquired from transferable plasmids or transposons. The acquisition of transferable elements may cause a dramatic increase in the erythromycin resistance, as in the case of two vancomycin-resistant strains. Additionally, erythromycin resistance genes could not be amplified from any of the strains using specific primer sets that were previously used for erythromycin resistance gene identification (Lina et al. 1999) (data not shown). Unsuccessful identification of the genetic determinants of erythromycin resistance can be attributed simply to primer design and PCR conditions; however, the existence of several antibiotic resistance mechanisms suggests that other genetic backgrounds may explain the phenotypic resistances. The twofold higher erythromycin resistance values might be a strain-specific property of Korean isolates. Other than the two vancomycin-resistant strains, all *Meju* strains were free from acquired antibiotic resistance. These strains could also be considered safe with regard to the lack of hemolytic activity and biofilm formation.

Biogenic amines are found in a variety of fermented foods, including beverages, meat products, and cheeses, and especially in protein-rich fermented fish products (Shalaby 1996; Sila Santos 1996). The major biogenic amines found in foods are cadaverine, histamine, putrescine, and tyramine. Excessive amounts of these compounds in food are undesirable, because they can result in severe toxicological effects in humans, such as hypertension, headache, diarrhea, and localized inflammation (Halasz et al. 1994). Biogenic amines are also precursors of carcinogens such as *N*-nitrosamine compounds. However, when consumed in small amounts, biogenic amines are not considered a serious risk. They are derived mainly from microbial decarboxylation of precursor amino acids produced during food fermentation. Therefore, the amount and types of biogenic amines formed in fermented food products are strongly influenced by the food composition, microbial flora, and environmental conditions, such as food processing and storage (Carelli et al. 2007). The possible production of biogenic amines by *Meju* isolates was estimated by measuring the conversion of histidine, lysine, ornithine, and tyrosine to histamine, cadaverine, putrescine, and tyramine, respectively. Less than 100 ppm of cadaverine and putrescine was produced by *E. faecalis* and *E. faecium*, while tyramine was present at levels of approximately 3000 ppm under the same conditions. Tyrosine decarboxylase is likely responsible for the high levels of tyramine produced by the *E. faecalis* and *E. faecium* strains, while the low-level production of cadaverine and putrescine could not be explained. The lack of specific amino acid decarboxylase genes could be used as a criterion for selecting safe starter strains if the food matrix contains high amounts of the specific precursor.

Other than the two vancomycin-resistant strains, none of the tested *Enterococcus* strains from *Meju* displayed the safety-related properties of antibiotic resistance, hemolytic activity, or biofilm formation. However, their potential for high tyramine production could limit their use as starters for *Meju* fermentation. Through this study, we reconfirmed the necessity of antibiotic resistance assessment of *Enterococcus* strains, regardless of their origins, before being used for food manufacture or as feed supplements, especially as the two vancomycin-resistant strains were isolated from *Meju*. Additionally, we showed that biogenic amine production should be assessed prior to selection of strains for soybean fermentation, as soybean is a protein-rich food material.

Salt tolerance of *E. faecalis* and *E. faecium* strains suggests that the species cannot proliferate in the fermentation of *Doenjang*, which is ripened at a 12 % NaCl concentration. This was previously mentioned in our analysis of the bacterial community during the *Doenjang* ripening process using a culture-dependent method (Jeong et al. 2014b), while *Enterococcus* species were also identified in *Doenjang* and *Meju* in culture-independent studies (Nam et al. 2012; Jung et al. 2014). This discrepancy between the analytical methods can be attributed to the differences between analyzed samples. However, the results of PCR-based bacterial community analyses are

known to have inherent biases introduced by the selective extraction of nucleic acids, selective amplification of the 16S rRNA gene, and the presence of dead cells. We propose that dead cells in the samples may be the major cause of *Enterococcus* species identification in culture-independent studies.

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