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Isolation of *Weissella* strains as potent probiotics to improve antioxidant activity of salted squid by fermentation

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Abstract The aim of this study was to enhance the antioxidant activity of salted squid by inoculation of two *Weissella* spp. strains (*W. cibaria* FB-069 and *W. viridescens* FB-077) isolated from traditional Korea salted squid. The safety and probiotic potential characteristics of these two strains were evaluated. The safety of these strains was analyzed based on hemolytic activity, mucin degradation, biogenic amino production, and resistance to antibiotics. These lactic acid bacteria showed probiotic potential, including resistance to gastrointestinal tract conditions, adhesion to Caco-2 cells, and aggregation. The low-salted squid fermented with *Weissella* strains had consistently higher antioxidant activity through changing their amino acid profiles. Therefore, *W. cibaria* FB-069 and *W. viridescens* FB-077 might be good candidates for fermentation of salted squid to develop functional food with enhanced health benefits.

Keywords Antioxidant · Probiotic · Salted squid · *Weissella*

Introduction

Naturally fermented food and beverages can be classically defined as functional foods with beneficial effects for the host (Stanton et al. 2005; Leroy and De Vuyst 2014). Most fermented foods contain a variety of microorganisms that might transform chemical constituents of food sources during fermentation, resulting in enrichment of substrates with vitamins, essential amino acids, bioactive compound, improvement of nutrient, inhibition of pathogen, and degrading toxic components (Leroy and De Vuyst 2014; Koo et al. 2016). The consumption of fermented food has beneficial effects, including health-promoting, anti-aging, anti-carcinogenic, and anti-obesity activities. In addition, it can prevent hypertension, cardiovascular diseases, gastrointestinal disorders, diabetes, osteoporosis, and cancer (Yang et al. 2014; Koo et al. 2016). However, the clinical evidences for health benefits of fermented foods in large and long-term prospective studies is very limited (Marco et al. 2017). In addition, most naturally fermented foods in different countries and regions of the world are still at home production under traditional conditions (Dolci et al. 2015).

Fermented seafood products have many unique forms across East Asia, Southeast Asia, and Europe. Ojingeojeot is one of the highly consumed seafood products in Korea. It is produced from squid, salted, and fermented for inhibition of spoilage (Koo et al. 2016). In a previous study, we have reported the antioxidant activity of fermented squid with soymilk additive (Akther et al. 2017). It has been shown that salted squid has high anticancer activity on liver hepatocellular carcinoma cell HepG2 (Lim et al. 2001). Until now, salted squid has mainly used as an additive in kimchi fermentation. Based on consumers' demand, salted squid has a great potential to be recognized as a nutritious and palatable food product globally. Therefore, efforts on research and development of salted squid are needed.

Lactic acid bacteria (LAB) are the main group of probiotic microorganisms with several healthy benefits on the host (Markowiak and Śliżewska 2017). The most commonly used probiotics are *Lactobacillus* and *Bifidobacterium* gena. The main selection criteria for probiotics are a long history of safe use and high genotypic stability (Sanders et al. 2010). However, only a few and specially selected strains are used in functional food (Markowiak and Śliżewska 2017). Among bacteria associated with fermented squid, dominant strains are *Bacillus*, *Staphylococcus*,

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and *Weissella*, followed by *Carnobacterium*, *Leuconostoc*, and *Pediococcus* (Koo et al. 2016). Some *Weissella* species can cause sepsis with other health-detrimental roles in human clinical infections, whereas other species of *Weissella* have beneficial roles in food preservation and key functions in food fermentation (Fairfax et al. 2014). It has been shown that some *Weissella* strains isolated from ojingeojeot possess resistance to gastrointestinal tract and bile juice with antimicrobial activity to *Listeria monocytogenes* (Kim et al. 2005). *Weissella cibaria* has an anti-inflammatory effect on dendritic cells and in animal models (Lim et al. 2017). Non-digestible polysaccharides from *Weissella* sp. have been used as prebiotics or for additive ingredients in food industries (Baruah et al. 2017; Kanimozhi et al. 2017). Some *Weissella* strains have been used in the preparation of functional foods. For example, the anti-obesity effect was observed in kimchi fermented with *Weissella koreensis* OK1-6 (Park et al. 2012). In addition, *Weissella* sp. mediated soymilk fermentation showed a significant increase in total isoflavone aglycones content compared to control (Chun et al. 2008).

In this paper, *Weissella* spp. isolated from the traditional salted squid was screened for their probiotics properties such as resistance to the gastrointestinal tract, antibiotic resistance, and their abilities of auto-aggregation and co-aggregation. We further evaluated the antioxidant activity of salted squid after inoculation with potential *Weissella* isolate as a starter culture.

Materials and Methods

Isolation of LAB strains from traditional salted squid

Twenty-two traditional homemade salted squid samples were collected from different cities in Jeollanam-do Province, Korea. All samples were collected in sterile tubes (15 mL) and kept in an icebox. After transporting to the laboratory, these samples were stored at −20 °C. Isolation of strains was finished within two days. For isolation of LAB strains, several dilutions of samples were spread onto De Man Rogosa Sharpe (MRS, Difco, Detroit, MI, USA) agar plates. After anaerobic incubation at 37 °C for 48 h, colonies with different morphotypes were collected in MRS broth supplemented with 25% glycerol and stored at −80 °C for further analysis.

Antioxidative activity

Each strain was cultured in MRS broth at 30 °C for 24 h. After incubation, the cell-free cultured broth was used to determine antioxidative activity. Aliquots (15 mL) of each medium were taken during fermentation (0, 4, 8, 20, 24, 32, and 44 h) to determine cell growth and antioxidant activity. The antioxidant capacity of the supernatant was determined by four different assays: DPPH (Brand-Williams et al. 1995), ABTS (Re et al. 1999), hydroxyl (Hagerman et al. 1998), and superoxide radical scavenging activity (Robak and Gryglewski 1988). Results were expressed as scavenging activity (%).

Strain identification

Total DNA was isolated using Genomic DNA Prep Kit (Solgent, Daejeon, South Korea) according to the manufacturer's instructions. PCR amplification of 16S rRNA gene was performed using the following primers: 27F, 5'-AGAGTTTGATCCTGGC TCAG-3'; and 1492R, 5'-GGTTACCTTGTTACGACTT-3'. The sequencing of the 16S rRNA gene was conducted by Bioneer Co. (South Korea). Sequence was analyzed for similarities by accessing data deposited at National Center for Biotechnology Information using BLAST program. A phylogenetic tree was constructed using closely related sequences showing high similarities in BLAST results followed by neighbor-joining phylogenetic tree construction using MEGA7 program.

Safety assessment

Fresh lactobacilli broth cultures (8.0-9.0 log CFU/mL) were streaked onto agar plates in triplicates for hemolytic reaction, mucin degradation, and biogenic amines production. After 48 h of incubation at 37 °C, plates were examined for activities. *Lactobacillus rhamnosus* GG (L.GG) ATCC 53103 and *S. aureus* KCCM 11335 strains were used as negative and positive controls, respectively. For hemolytic activity, Columbia agar plates (DB, Difco, Detroit, MI, US) containing 5% (w/v) defibrinate sheep blood were used. Mucin degradation was determined on 0.3% porcine stomach mucin supplied agarose medium (DB, Difco) with or without glucose. Biogenic amine production of the isolate was determined using decarboxylase medium (DB, Difco) containing 1% separate amino acids such as L-tyrosine, L-histidine, L-ornithine, L-arginine, L-phenylalanine, L-lysine, or L-tryptophan.

Disc diffusion assay

The antimicrobial activity was determined by agar disc diffusion assay based on standard protocol of Clinical and Laboratory Standards Institute (CLSI 2015). *Bacillus cereus* KACC 11240, *Shigella boydii* KACC 10792, *Listeria monocytogenes* KACC 10764, *Yersinia enterocolitica* subsp. *enterocolitica* KACC 15320, *Escherichia coli* K99 KCTC 261, *Salmonella enterica* serotype Choleraesuis KCTC 2932, *Salmonella enterica* serovar Typhi KCTC 2514, *Salmonella enterica* subsp. *enterica* serovar Gallinarum KCTC 2931, and *Staphylococcus aureus* KCCM 11335 were used as pathogen strains. The diameter of the clean zone around each of the discs was taken as a measure of the antimicrobial activity.

Antibiotic susceptibility

Broth microdilution assay was applied to determine antibiotic susceptibility of each strain (Patel et al. 2014). Each well contained 100 µL of 5.0 log CFU/mL of inoculum and 100 µL of serially diluted (two-fold) antibiotics. Penicillin, ampicillin, vancomycin, streptomycin. kanamycin and tetracycline (Sigma-Aldrich, St

Louis, MO, USA) were chosen according to the list proposed by the Clinical and Laboratory Standard Institute (CLSI). Plates were incubated at 37 °C and the minimum inhibitory concentration was evaluated at 24 h after incubation.

Assessment of probiotic potential

Tolerance to simulated GI tract condition test was performed by successively exposing test strain to oral-gastric-intestinal simulated juice as described by Damodharan et al. (2015). Bacterial viability at the end of each stress was monitored using LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Eugene, Oregon, USA) following the manufacturer's protocol.

Adhesion assay was performed using human colonic cell line Caco-2 (passages 29-31) obtained from KCTC (Korea). Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) which was routinely changed every two days until a confluent monolayer was obtained. After washing twice with PBS (pH 7.2), fresh DMEM was resuspended in serum- and antibiotic-free medium with 1 mL of bacterial suspension (8.0 log CFU/mL). After incubation at 37 °C with 5% CO_2 for 1 h, cells were washed twice with PBS to remove non-attached bacterial cells. Cells were then detached from each well with 1 mL of 1% (v/v) Triton X-100. Bacterial adhesion (%) was determined after plating serial dilutions of the suspension onto MRS plates followed by incubation at 37 °C for 24 h.

Specific cell-cell interactions were determined according to a published method (Xu et al. 2009). Briefly, bacterial cells were cultured overnight, centrifuged at 5000×g for 10 min at 4 °C, and washed with PBS. The cell pellet was resuspended in PBS (mixed by pipetting) to reach a final concentration of 8.0 log CFU/mL. The absorbance of bacterial suspension at 600 nm was measured (A0). The suspension was then incubated at 37 °C for 5 h. The absorbance of the supernatant was then measured at 600 nm (An, n=1, 2, 3, 4, and 5). Percentage of auto-aggregation was calculated as follows:

$$\text{Auto-aggregation (\%)} = (A0 - An/A0) \times 100$$

Isolate and pathogen suspensions were prepared as described earlier for auto-aggregation followed by mixing at equal volume. They were then incubated at 37 °C for 5 h. Absorbance values of the supernatant before and after incubation were measured at 600 nm. Percentage of co-aggregation was calculated using the following formula:

$$\text{Co-aggregation (\%)} = [1 - A_m/(A_i + A_p)/2] \times 100$$

where A_p and A_i were absorbance values of the pathogen and isolated strains suspension in control tube and A_m was absorbance value of mixed bacterial suspension after incubation.

Preparation of salted squid fermentation

Fresh salted squid samples were supplied by Greenmin Food Co., Yeosu, Korea. In brief, the culture was prepared using 3:1 (w/w) fresh squids and seasoning (hot pepper flakes, salt). The salted squid was inoculated with about 7.0 log CFU/mL of isolated strains. The culture was mixed well with the bacteria and quiescently fermented at 4 °C for 0, 1, and 2 months. The non-fermented salted squid was used as control. All fermentations were carried out in triplicates.

Antioxidant capacity of fermented squid samples

For extraction of salted squid, 25 g of fermented sample was homogenized with 250 mL of ethanol : DMSO : water (70:30:1, v/v/v) at 24,000 rpm and stirred for 1 h at 25 °C. The sample was then centrifuged at 8,000×g for 10 min to collect the supernatant. Antioxidant capacity of the supernatant was then determined as described above.

Amino acid analysis

Salted squid (1 g) was hydrolyzed with 100 mL of 6 M HCl in sealed ampoules in an oven at 110 °C for 22 h. The mixture was then passed through a 0.45-μm membrane filter and injected into an automatic amino acid analyzer (S-433, SYKAM GmbH, Munich, Germany).

Statistical analysis

All experiments were performed in triplicates. Results are presented as mean ± SD (standard deviation). One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were performed using SPSS software ver. 22, (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $p < 0.05$.

Results and Discussion

Selections of strains

The safety assessment is the first criterion of probiotics to ensure that its products do not pose any risk to consumer health. Thus, these isolates were selected based on international guidelines for evaluation of probiotic potential provide by FAO/WHO (2002). If bacteria have hemolytic activity, they could be virulent and cause disease despite host resistance mechanisms. The production of a microbial enzyme capable of degrading mucin or producing biogenic amines might favor mucosal invasion by pathogens and other toxic agents indicative of food spoilage. Therefore, these properties are not considered desirable features for probiotic strains. We investigated a collection of ten LAB isolated from the traditional salted squid to determine their hemolytic ability, mucin degradation, and biogenic amine producing activities.

Antioxidant activity of lactic acid bacteria

The antioxidant activities of ten LAB isolates from salted squid were determined based on their DPPH, hydroxyl radical, and superoxide radical scavenging activities. Results are shown in Fig. 1. Among these ten strains tested, FB-069 had the highest

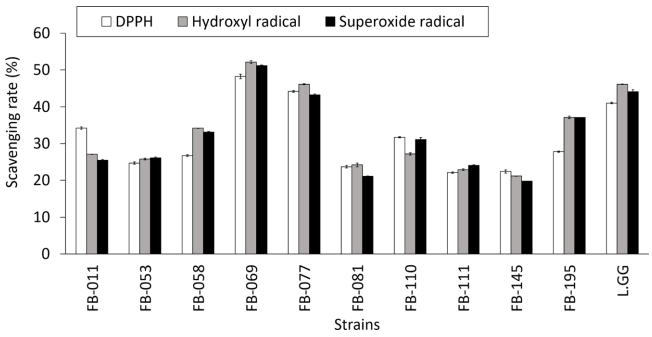

Fig. 1 Scavenging activities on DPPH, hydroxyl radicals, and superoxide radical by intracellular cell-free extracts of isolates (10^9 CFU/mL) incubated at 37 °C for 18 h. Each value is expressed as mean ± SD (n =3)

scavenging activities for DPPH free radical, hydroxyl radical, and superoxide radical, with inhibition rates of 48.2, 52.1, and 51.2%, respectively, at 9.0 log CFU/mL. FB-077 strain also showed high antioxidant activity, similar to *Lactobacillus rhamnosus* GG. Antioxidative activities of LAB such as *L. casei*, *L. fermentum*, L.GG, and *Streptococcus thermophilus* have been reported in previous studies (Kim et al. 2005; Abushelaibi et al. 2017). The FB-69 strain was most similar to *Weissella cibarica*, whereas the FB-077 strain was clarified within *W. viridescens* based on 16S rRNA gene BLAST analysis (>99%) and phylogenetic tree (Fig. 2).

Antimicrobial activity and antibiotic susceptibility

Two potential strains showed different antimicrobial activities as shown in Table 1. Both strains effectively inhibited the growth of Gram-negative *E. coli* and *Salmonella* spp. at various degrees. They showed the highest antimicrobial activity against *S. gallinarum* (diameter of the inhibition zone >8 mm).

The resistance of microbe to antibiotics is a negative characteristic when selecting a probiotic candidate because of the possibility of horizontal gene transfer of antibiotic-resistant genes among intestinal microbiome which might cause a serious risk for the treatment of infected patients (Ochman et al. 2000; Salyers et al. 2004). These *Weissella* strains were found to be resistant to kanamycin (with minimum inhibitory concentration (MIC) of ≥128 μg/mL), vancomycin (MIC ≥64-128 μg/mL), and tetracycline (MIC ≥32 μg/mL) (Table 1). Until now, there are no standard methods or interpretation criteria of antimicrobial susceptibilities established for *Weissella* spp. by the Clinical and Laboratory Standards Institute (Patel et al. 2014). However, it has been reported that *Weissella* spp. are intrinsically sensitive to ampicillin but resistant to kanamycin and vancomycin (Lee et al. 2012; Medford et al. 2014).

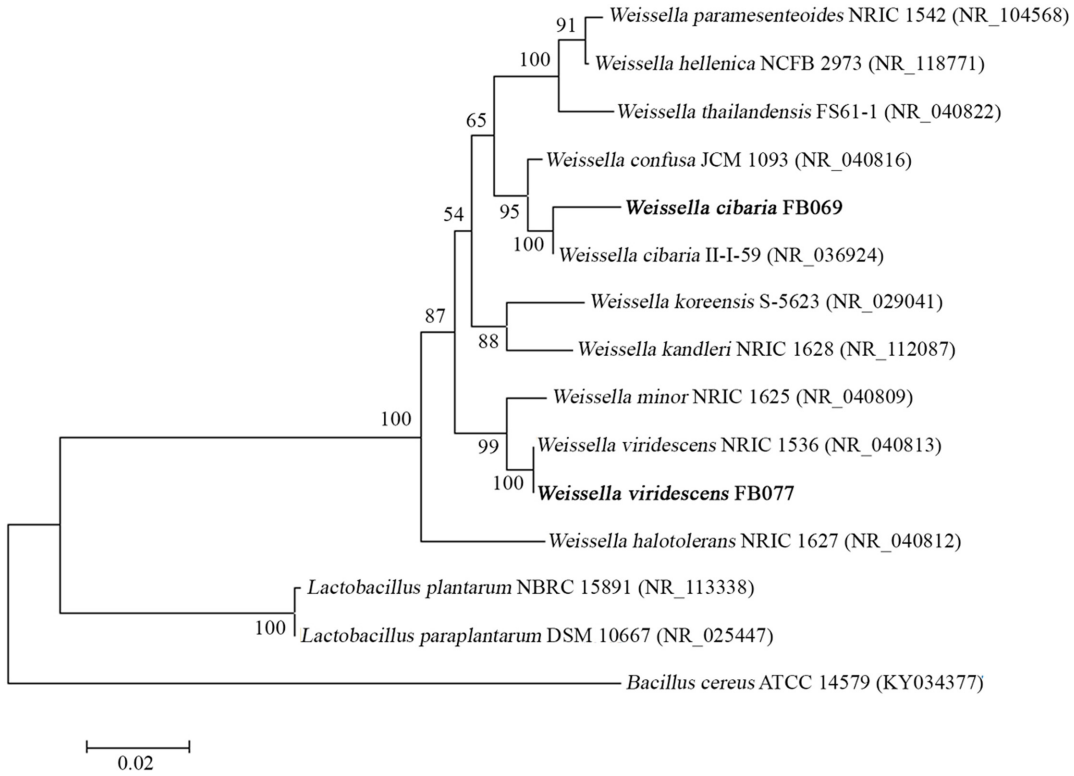

Fig. 2 Phylogenetic tree constructed based on 16S rRNA gene sequence of strains FB069, FB077 isolated from salted shrimp. The GenBank accession number is indicated in parenthesis

Table 1 Antimicrobial activity of cell free supernatant broth of isolates against common pathogens, antibiotic resistance (MIC) and safety assessment

		FB069	FB077			FB069	FB077
Pathogenic strains	*B. cereus*	+	+	MIC (μg/mL)	Penicillin	4	4
	E. coli K99	++	++		Ampicillin	0.5	0.5
	L. monocytogenes	+	++		Vancomycin	64	128
	S. Gallinarum	+++	+++		Streptomycin	8	8
	S. aureus	++	+		Kanamycin	128	128
	S. Choleraesuis	++	++		Tetracycline	32	32
	S. Typhi	+	++	Bioamine production		ND	ND
	S. boydii	+	++	Hemolytic activity		ND	ND
	Y. enterocolitica	+	+	Mucin degradation		ND	ND

+: 4 mm; ++: 8 mm; +++: zone >8 mm
ND: non-detected

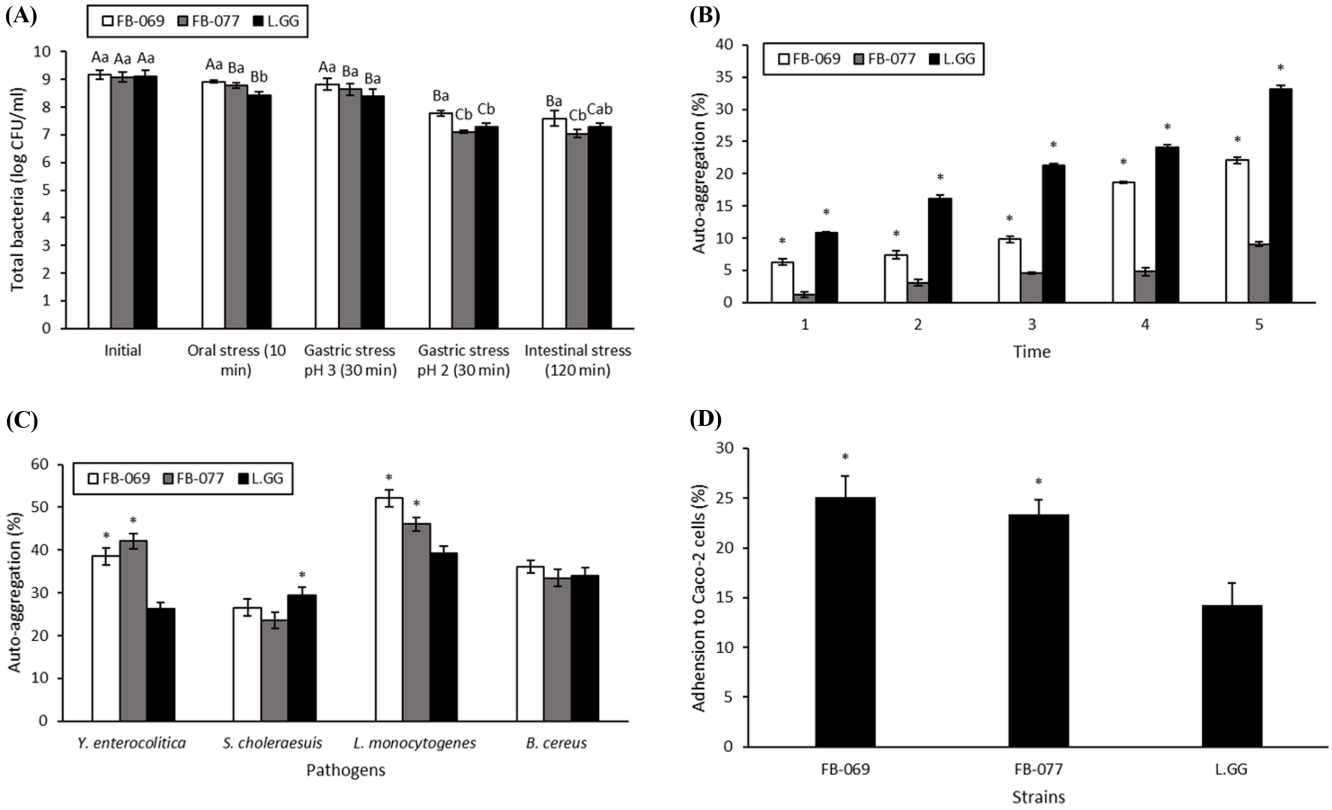

Fig. 3 Probiotic potential characteristics of isolates. (A) Tolerance of viable bacterial count different pH in gastrointestinal. Data are expressed as mean ± SD (n =3). The uppercases indicate significant difference in different pH values within the same isolate. The lowercases indicate significant differences among different isolates within the same pH (p <0.05)

Probiotic characteristics

There was a significant decrease ($p \leq 0.05$) in the population of all strains evaluated after consecutive exposure to gastric and small intestine conditions using *in vitro* model (Fig. 3A). *W. viridescens* and L.GG strains showed similar population at the end of the assay, with average survival ratio of 77.4 and 79.9%, respectively, based on cell count reduction. Similar to previous observation, LAB showed good resistance to oral and intestinal environment. However, in the gastric tract, most strains were killed under extremely low pH (Lee et al. 2012; Ellis et al. 2016). The survival ability of LAB with acid stress depends on exogenous conditions which can affect intracellular pH and cell membrane functionality and induce stress-response proteins of microbes (Wu et al. 2014).

Results for the percentage of auto-aggregation of the two *Weissella* isolates and reference strain are shown in Fig. 3B. After 5 h of auto-aggregation, *W. viridescens* FB-069 showed the least auto-aggregative ability (9.1±0.31%, $p \leq 0.05$) whereas L.GG showed the highest (33.2±0.53%). Auto-aggregation can enhance

gastrointestinal persistence of a probiotic *in vivo* as well as its colonization *in vitro* (Cesena et al. 2001). Auto-aggregation results of our study are consistent with results reported by others (Angmo et al. 2016; Abushelaibi et al. 2017).

Results of co-aggregation between *Weissella* strains and *Y. enterocolitica*, *S. choleraesuis*, *L. monocytogenes*, or *B. cereus* are shown in Fig. 3C. Compared to L.GG, *W. cibaria* showed higher co-aggregation abilities with *Y. enterocolitica* (38.5%) and *L. monocytogenes* (52.1%). In general, the co-aggregation ability of *Weissella* genus was strains-specific. The *Weissella* strains had lower co-aggregation abilities with *S. choleraesuis* Gram-negative pathogens compared to other tested pathogens.

Adhesion rates of both *Weissella* isolates were higher compared to the reference L.GG strain ($p \leq 0.05$) (Fig. 3D). Although adhesion of microbes on intestinal epithelial cells has been used as a strategy to select probiotics, a wide range of adhesion abilities of probiotics to human cells have been reported recently (Yadav et al. 2015; Ellis et al. 2016). Moreover, results obtained from adhesion tests are different from the reality *in vivo* because of the sensitive and specific human GI tract may change the strain's adhesion ability (Vinderola et al. 2017).

Antioxidant capacity of salted squid

In general, fermentation of food with LAB to improve nutritional quality and health benefits of fermented foods has been recognized for a long time (Koo et al. 2016). In the present study, DPPH and hydroxyl radical scavenging capacities of salted squid fermented with *Weissella* spp. were significantly increased compared to salted squid without inoculation of bacteria after two months of fermentation (Table 2). DPPH inhibition was slightly increased 18.7 and 20.2%, respectively, after two months of incubation. The similar pattern of DPPH value was similar to that of hydroxyl radical value for both *Weissella* strains. Those salted squids inoculated by *Weissella* bacteria showed significantly ($p < 0.05$) higher hydroxyl radical scavenging activity compared to control fermentation. However, ABTS free radical scavenging capacity of all salted squid sample was not increased even after two months of fermentation. *W. cibaria* FB-069 strain showed higher antioxidant effect on superoxide radical scavenging activity of salted squid. However, the antioxidant effect of salted squid was not significantly changed after inoculation with *W. viridescens* FB-077. Lee and Kim (2012) have studied the antioxidant activity of salted squid extracted from low-salted fermented squid and commercial high salted squid and found that hydroxyl radical scavenging activities are in range of 20.4 to 25.2%. In another study, incorporation of *W. confusa* LK4 at a dose of 7.0 log CFU/mL showed an increase of 16.9% in radical scavenging activity and a decrease of 19.41% in Korean leek fermentation compared to spontaneous fermentation (Yang et al. 2014). They confirmed that the higher antioxidant in fermented samples could be due to more production of bioactive compounds such as flavonoid, poly-phenolic compounds, and amino acids.

Nutritional analysis

Amino acid profiles of salted squid changed between the beginning and after 2-month of fermentation with the two isolates of *Weissella* (Table 3). *Weissella* spp. inoculated in salted squid were found to harbor different dominating amino acids such as proline, glutamine, arginine, threonine, and lysine. They appeared to remain stable over time. Threonine, glutamine, and isoleucine were increased significantly while serine, glycine, and alanine were decreased significantly during both fermentation. The total essential amino acid content was initially 37.1%. It was increased ($p < 0.05$) to 40.0 and 41.3% after two months of fermentation with *W. cibaria* FB-069 and *W. viridescens* FB-077, respectively. These percentages are adequate as ideal protein food based on WHO criteria: 26.5% for infants, 22.6% for children, and 21.7% for adults (WHO 2007). Total neutral amino acids and alkaline amino acids content showed slight change but significant ($p < 0.05$) decrease in both fermentation. Predicted efficiency ratio (P-PER) is one of the quality parameter used for protein evaluation. P-PER values of *W. cibaria* FB-069 and *W. viridescens* FB-077 salted squid were 1.3 and 1.6, respectively. They are close to the P-PER value of 1.21 for cowpea and 1.22 for tuber anchote (Salunkhe

Table 2 Antioxidant activities of the extracts from salted squid (SS)

Time (month)	Fermentation	Inhibitory activity (%)			
		DPPH	ABTS	Hydroxyl radical	Superoxide
0	SS + FB-069	22.1±0.23^{defh}	23.9±0.31^{de}	23.7±0.47^{def}	17.1±0.35^{i}
	SS + FB-077	23.4±0.25^{def}	24.1±0.51^{de}	24.1±0.15^{de}	18.1±0.51^{hi}
	SS without fermentation	23.1±0.69^{def}	24.1±0.54^{de}	24.4±0.61^{de}	17±0.56^{i}
1	SS + FB-069	28.1±2.31^{c}	31.1±0.06^{b}	30.7±2.93^{b}	20.9±0.64^{fh}
	SS + FB-077	27.2±0.61^{c}	32.5±0.05^{b}	30.8±4.12^{b}	21.1±0.64^{efh}
	SS without fermentation	24.1±0.64^{de}	31.5±0.91^{b}	26.4±0.61^{cd}	20.1±0.22^{hi}
2	SS + FB-069	31.1±0.25^{b}	32.4±0.25^{b}	35.2±0.81^{a}	24.5±2.34^{de}
	SS + FB-077	31.5±0.71^{b}	32.1±0.06^{b}	36.1±0.9^{a}	22.1±0.12^{defh}
	SS without fermentation	26.2±2.32^{cd}	32.6±0.1^{b}	31.1±5.22^{b}	20.3±0.9^{hi}

Data were expressed as mean±standard deviation. Different superscript lowercase letters indicated statistical significance ($p < 0.05$)

Table 3 Total amino acid profiles of salted squid (SS)

Amino acid	Fermentation period		
	0 month	2 months	
	SS without fermentation	SS + FB-069	SS + FB-077
Asp	8.4±0.26^{Bb}	9.4±0.21^{Ad}	8.5±0.22^{Bd}
Thr	3.32±0.21^{Bcd}	7.2±0.42^{Ae}	9.1±0.11^{Acd}
Ser	4.21±0.63^{Bc}	3.6±0.31^{Ah}	2.4±0.51^{Aj}
Glu	4.31±1.31^{Bc}	11.2±0.42^{Ab}	9.4±0.57^{Ac}
Gly	5.1±0.66^{Ac}	2.5±0.15^{Cj}	3.5±0.12^{Bi}
Ala	8.1±0.91^{Ab}	3.4±0.38^{Bh}	3.6±0.51^{Bi}
Cys	1.2±0.06^{e}	1.3±0.51^{k}	1.6±0.34^{j}
Val	4.8±0.11^{c}	4.6±0.51^{g}	4.7±0.26g^{h}
Met	2.1±0.12^{Bde}	2.6±0.42^{ABij}	3.6±0.87^{Ai}
Ile	4.9±0.33^{Bc}	5.4±0.13^{Af}	5.7±0.13^{Af}
Leu	8.9±0.22^{Aab}	4.6±0.01^{Bg}	5.4±0.71^{Bfg}
Tyr	3.5±0.38^{cd}	3.1±0.56^{hi}	3.5±1.11^{i}
Phe	4.1±0.19^{c}	4.2±0.21^{g}	4.3±0.25^{hi}
Lys	7.2±0.31^{Bb}	9.8±0.34^{Ad}	6.8±0.21^{Be}
His	1.8±0.52^{de}	1.6±0.24^{k}	1.7±0.51^{j}
Arg	10.3±0.11^{b}	10.4±0.21^{c}	10.6±0.34^{b}
Pro	17.8±0.39^{Aa}	15.1±0.11^{Ba}	15.6±0.32^{Ba}
Total essential amino acids	37.12±0.25	40±0.29	41.3±0.38
Total neutral amino acids	55.8±0.37	42.4±0.24	46.4±0.4
Total acidic amino acids	12.71±1.57	20.6±0.63	17.9±0.79
Total alkaline amino acids	19.3±0.31	21.8±0.26	19.1±0.35
Total sulfur amino acids	3.3±0.09	3.9±0.47	5.2±0.61
Total aromatic aminon acids	9.4±0.21	8.9±0.34	9.5±0.25
Leu/Ile ratio	1.8±0.11	0.8±0.12	0.9±0.41
P-PER	3.21±0.31	1.29±0.12	1.62±0.51

Data were expressed as mean ± standard deviation. The superscript uppercases indicate significant difference in each amino acid. The lowercases indicate significant differences among the same salted squid sample (p <0.05)

and Kadam 1989; Ayalew et al. 2017). However, they are lower than the P-PER value of 2.5 for reference casein from cow's milk protein (WHO 1985). The amino acid imbalance is highly related to the development of serious diseases because of decreases of activity levels, food consumption, and absolute protein intake (WHO 2007). Taken together, our results showed that the nutritional quality of salted squid was improved through fermentation with *Weissella* strains.

The results of this study could be very useful to the selection of beneficial bacteria to develop low-salted squid as functional food or food additive with health benefits. In the present work, the safety and probiotics properties of *W. cibaria* FB-069 and *W. viridescens* FB-077 were identified. Both strains could enhance the bioactivity of salted squid. Overall, the antioxidant activity increased with the increasing fermentation time with both strains. They showed highly effective antioxidant properties, especially scavenging abilities for DPPH and hydroxyl radicals. These results suggest that *Weissella* strains may be potent alternative natural bacteria as starter culture for low-salted squid. The future investigations should explore the efficacy of *Weissella* genus in fermented process and *in vivo* experimental model.

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