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Functional Properties of Squid By-products Fermented by Probiotic Bacteria

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Abstract The effects of probiotic bacteria on the functional properties of squid by-products were investigated during fermentation. *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Pediococcus acidilactici* were used to ferment the squid by-products for 96 hr at 37°C. The numbers of all probiotics increased to 10^7-10^8 CFU/g after 96 hr fermentation. No substantial pH changes were observed. *L. rhamnosus* and *P. acidilactici* showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) secreted from B cells increased after adding the extracts of probiotic-fermented squid by-products. The human NK cells were grown well in the B cell-growing broth cultured with the extracts of squid by-products fermented by *L. rhamnosus* and *P. acidilactici*. Trimethylamine (TMA) and dimethylamine (DMA) contents were significantly decreased after probiotic-fermentation. Therefore, *L. rhamnosus* GG and *P. acidilactici* can be used for the fermentation of squid by-products and their use would provide benefits in functional food products.

Keywords: probiotic bacterium, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trimethylamine, dimethylamine, squid by-product, interleukin-6, tumor necrosis factor-α

Introduction

Squid belongs to the class of Cephalopod, which is widely distributed in the Atlantic Ocean, the Indian Ocean, and the Pacific Ocean. The worldwide squid production was 2,827,700 tons in 2006 (1). During the processing of squids for human consumption, approximately 30% of the squid including the head, liver, skin, and ink bursa are wasted; the total amount of wastes can be approximately 565,540 to 848,310 tons per year. Squid by-products contain protein, fatty acids, and collagen. Squid ink containing a heat-resistance peptide polysaccharide has anti-ulcer function and anti-tumor activity (2-4). The protamine has antimicrobial activity, which can inhibit the growth of *Listeria monocytogenes* and *Bacillus cereus* (5). Accordingly, the squid by-products can be used for various industrial applications. However, the squid by-products are used as feed ingredients and mostly wasted, leading to a tremendous waste of fishery resources and a potential danger of environmental pollution.

Probiotics, defined as 'live microorganisms which beneficially affect the host animal by improving its intestinal microbial balance', are microorganisms that are considered non-pathogenic, safe, and health beneficial (6,7). Various microorganisms have been reported as probiotics such as lactobacilli, bifidobacteria, and enterococci (8). Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used for fermented dairy products or food supplements because of their beneficial function and safety (9-11). Other microorganisms, including *Pediococcus acidilactici* and *Pediococcus. pentosaceus,* are also used as probiotics because they can produce bacteriocins (12-14). Hence, the use of probiotic organisms to ferment the squid by-products may not only improve the nutrient profile but may also provide beneficial health effects during the biological fermentation process. However, there are few reports regarding to fermenting the by-products from squid processing with probiotic cultures. To the best of our knowledge, it would be the first approach to investigate the possibility of the use of the by-products from squid processing as fermentation substrates for probiotic cultures. Therefore, the objectives of this study were to screen proper probiotic cultures for the fermentation of by-products from squid processing and investigate the potential of using the fermented squid by-products as a source of functional ingredients.

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Materials and Methods

Bacterial strains and culture condition *Lactobacillus paracasei* (ATCC 25598), *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* ADH, and *Bifidobacterium longum* B6 were kindly provided by Dr. Azlin Mustapha of the Department of Food Science at the University of Missouri-Columbia, USA. *Lactobacillus brevis* (KACC 10553), *Lactobacillus casei* (KACC 12413), and *Pediococcus acidilactici* (KACC 12307) were obtained from the Korean Agricultural Culture Collection (KACC, Suwon, Gyeonggi, Korea). All bacteria were anaerobically cultivated in de Man, Rogosa, and Sharpe (MRS, Difco, Detroit, MI, USA) broth supplemented with 0.05% cysteine hydrochloride at 37°C for 24 hr. After cultivation, the cultures were centrifuged at 4,000×g for 15 min (Sorvall RC-5; Block

Scientific, Nutley, NJ, USA) and washed twice in sterile 0.85% NaCl. The pellets were resuspended to 0.1% sterile buffered peptone water (BPW), and the harvested cells were diluted to approximately 10^8 CFU/mL in BPW for inoculation into squid by-product.

Sample preparation and fermentation By-products from squid processing, including cecum, intestine, and vena cava, used in this study were obtained from a local seafood processing company (HUDEM, Gangneung, Gangwon, Korea). The squid by-product mixtures were thoroughly homogenized for 3 min at 25°C using a mixer (HMF-560; Hanil Electric Co., Ltd., Seoul, Korea). Each probiotic culture was inoculated at approximately 10^6 CFU/g into the mixture. The inoculated samples and control (no inoculation) were fermented at 37°C for 96 hr in a GasPak anaerobic system (BBL, Cockeysville, MD, USA) with Anaerogen (Oxoid, Ltd., Basingstoke, Hampshire, UK).

Microbial analysis Pour plating was used to determine the viable counts. The fermented samples were serially (1:10) diluted with 0.85% NaCl and pour-plated with MRS agar. The agar plates were incubated for 48 hr at 37°C to determine the populations of probiotic bacteria. The experiments were performed in duplicate from 3 replicates.

pH measurement Each fermented sample was homogeneously mixed in sterilized plastic bags. The pH values of all samples were measured after 0 and 96 hr of fermentation using a pH meter (Fisher Scientific, St. Louis, MO, USA).

Cell cultures The human B cell Raji (ATCC No. CC-86) and NK-92MI cell (ATCC No. CRL-2408) lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The B cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich), penicillin (100 U/mL; Sigma-Aldrich), and streptomycin (100 μg/mL; Sigma-Aldrich) in the incubator (Sanyo Electric Biomedical Co., Ltd., Moriguchi, Osaka, Japan) with 95%(v/v) humidified air and 5%(v/v) CO_2 at 37°C. The NK-92MI cell line was cultured with minimum essential medium (MEM) (2.0 mM L-glutamine, 0.2 mM myoinositol, 20 mM folic acid, 0.1 mM 2-mercaptoethanol, 12.5% FBS, and 12.5% horse serum; Gibco, Grand Island, NY, USA) in the incubator (Sanyo Electric Biomedical Co.) with 5% CO_2 at 37°C.

DPPH assay The antioxidant activity of fermented supernatants was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (15). One mL of 0.2 mM DPPH radical solution in 95% ethanol was prepared and mixed with 2 mL of fermented supernatant. The mixture was incubated for 30 min at 25°C and measured the absorbance at 517 nm. The scavenging activity of DPPH radical was calculated by the following equation;

$$\text{DPPH scavenging activity (\%)} = [1 - (A_1/A_0)] \times 100$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance of fermented supernatant.

Cytokine assay The secretion of cytokines was determined by using interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) kits according to manufacture's instruction (Chemicon; Temecula, CA, USA). Briefly, approximately 2.0×10^4 cells/mL of B cells was cultured for 24 hr in 24-well plates (37°C, 5% CO_2). The cultured cells were further cultured for 6 days after adding 100 mL of fermented supernatants. The samples were centrifuged at 10,000×g (VS-15000N; Vision, Bucheon, Gyeonggi, Korea) for 10 min. The supernatants were used to measure the absorbance at 450 nm using a microplate reader.

Natural killer (NK) cell proliferation The human B cells were subcultured with 50 mL fermented supernatant in T-25 flask and centrifuged at 10,000×g for 10 min (VS-15000N; Vision). The supernatant of B cell culture was collected. NK-92MI cell line (approximately 5.0×10^4 cells/mL) was mixed with 100 μL of the supernatant of B cells and cultured at 24-well plates for 5 days. The number of NK-92MI cells was determined on a cell counter.

Dimethylamine (DMA) and trimethylamine (TMA) determination DMA and TMA were analyzed using gas chromatography (GC, HP 5890 Series; Hewlett Packard Palo Alto, CA, USA) equipped with a flame ionization detector and fitted with a WCOT fused silica column (60 m×0.32 mm i.d.×0.20 mm film thickness; Varian, Inc., Lake Forest, CA, USA) coating CP-Volamine (No. CP7447; Varian, Inc., Palo Alto, CA, USA). DMA and TMA were extracted using volatile amine extraction method with slight modification (16). The fermented supernatant (10 mL) were homogenized with 15 mL of 0.6 N perchloric acid and mixed with 50% KOH. The mixture was incubated at 60°C for 10 min. The amount of sample injected was 1 mL. Injector and detector temperatures were 75 and 230°C, respectively. Hydrogen was used as carrier gas at 30 mL/min with a pressure of 16.7 psi. Reference standards of DMA and TMA were prepared. Dilutions and peak areas were used to establish standard curves. DMA and TMA were identified by the relevant retention times, which were corrected with reference spectra established from reference standards.

Statistical analysis The Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. The general linear model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences among treatments or incubation times were calculated by Fisher's least significant difference (LSD) at $p<0.05$.

Results and Discussion

Functional food has received much attention in recent year because of its health-promoting and potential disease-preventing properties. The discovery of novel bioactive compounds is in high priority. In this study, the probiotic-fermentation approach has been found to provide several benefits, including the free radical scavenging activity, the potential immunomodulating activity, and the reduction of biogenic amines. Therefore, the positive results produced

Table 1. Growth of probiotic bacteria and changes in pH during the fermentation of squid by-products

Strain	Viability (CFU/g)		pH[1)]	
	0 hr	96 hr	0 hr	96 hr
B. longum B6	1.45×10^6	1.74×10^7	6.19[a]	6.13[d]
L. acidophilus ADH	1.23×10^6	4.17×10^8	6.19[a]	6.37[c]
L. brevis	1.02×10^6	1.66×10^8	6.19[a]	6.47[a]
L. casei	1.95×10^6	1.26×10^8	6.19[a]	6.04[e]
L. paracasei	2.04×10^6	4.57×10^7	6.19[a]	6.07[e]
L. rhamnosus GG	1.74×10^6	2.51×10^8	6.18[a]	6.12[d]
P. acidilactici	1.41×10^6	6.03×10^8	6.19[a]	6.43[b]

[1)a-e]Means with different subscript letters within a column are significantly different at $p<0.05$.

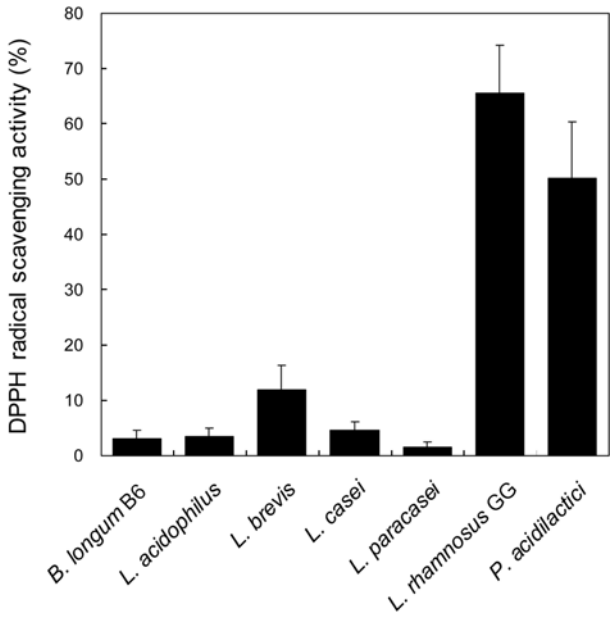

Fig. 1. DPPH radical scavenging activity of squid by-products fermented for 96 hr at 37°C by different probiotic bacteria.

in this study highlight the potential application of the probiotic-fermentation which is valuable and desirable for utilizing by-products from squid processing.

Screening of probiotic bacteria for use in fermentation of by-products from squid processing The growth of selected probiotic bacteria and the change in pH during the fermentation of squid by-product are shown in Table 1. The populations of *P. acidilactici*, *L. acidophilus* ADH, and *L. rhamnosus* GG increased to 6.03×10^8, 4.17×10^8, and 2.51×10^8 CFU/mL, respectively, after 96 hr fermentation. No significant changes in the pH were observed after 96 hr fermentation. However, the pH values of *B. logum* B6, *L. casei*, *L. paracasei*, and *L. rhamnosus* GG slightly decreased after 96 hr fermentation, while those of *L. acidophilus*, *L. brevis*, and *P. acidilactici* slightly increased. The production of organic acids during the fermentation is more likely responsible for the pH decline. DPPH activities for the probiotic bacterial fermented by-products from squid processing are shown in Fig. 1. The fermented

Table 2. Secretion of IL-6 from B cells cultured in adding the extracts of squid by-product fermented by probiotic bacteria

Time (day)	IL-6 (pg/10^4 cells)[1)]		
	Control	*L. rhamnosus* GG (10^{-4} pg/cell)	*P. acidilactici*
1	1.05±0.21[c,y]	2.16±0.22[d,x]	2.06±0.23[e,x]
2	1.41±0.15[c,y]	3.89±0.30[c,x]	3.28±0.11[d,x]
3	2.30±0.28[b,y]	4.06±0.23[c,x]	3.49±0.25[d,x]
4	3.51±0.15[a,z]	6.58±0.25[b,x]	4.74±0.20[c,y]
5	3.73±0.24[a,z]	7.57±0.33[a,x]	6.68±0.17[b,y]
6	3.92±0.26[a,y]	7.27±0.10[a,x]	7.16±0.08[a,x]

[1)]Means with different subscript letters within a row (x-z) and a column (a-e) are significantly different at $p<0.05$.

Table 3. Secretion of TNF-α from B cells cultured in adding the extracts of squid by-product fermented by probiotic bacteria

Time (day)	TNF-α (pg/10^4 cells)[1)]		
	Control	*L. rhamnosus* GG (10^{-4} pg/cell)	*P. acidilactici*
1	0.70±0.28[c,y]	1.65±0.21[f,x]	1.58±0.25[f,x]
2	0.77±0.13[c,z]	3.33±0.18[e,x]	2.71±0.15[e,y]
3	1.09±0.21[b,y]	3.99±0.16[d,x]	3.99±0.16[d,x]
4	1.47±0.18[b,z]	6.05±0.21[c,x]	4.75±0.22[c,y]
5	2.34±0.33[a,y]	6.96±0.22[b,x]	6.43±0.18[b,x]
6	2.71±0.16[a,y]	7.73±0.24[a,x]	7.42±0.11[a,x]

[1)]Means with different subscript letters within a row (x-z) and a column (a-f) are significantly different at $p<0.05$.

supernatants of *L. rhamnosus* GG and *P. acidilactici* showed much higher antioxidant activity after 96 hr fermentation than those of other probiotic bacteria, indicating that the fermented supernatants acted as good antioxidants.

***In vitro* effect of probiotic bacterial fermented squid by-products on the production of IL-6 and TNF-α** The secretion of IL-6 and TNF-α from the human B cells was tested to investigate the effect of selected probiotic bacterial fermentation on the immune systems (Table 2 and 3). Compared to the control, the amounts of IL-6 and TNF-α were significantly ($p<0.05$) increased throughout the cultivation when B cells were exposed to the probiotic-fermented extracts. The amounts of IL-6 secreted from the B cells cultured with *L. rhamnosus*- and *P. acidilactici*-fermented squid by-product extracts increased to 7.27 and 7.16 pg/10^4 cells after 6 days of cultivation, respectively (Table 2). The amounts of TNF-α secreted from the B cells cultured with *L. rhamnosus*- and *P. acidilactici*-fermented squid by-product extracts increased to 7.73 and 7.42 pg/10^4 cells after 6 days of cultivation, respectively (Table 3). The modulation of the immune response might be attributed to the enhanced production of cytokines. IL-6 is secreted by various cell types such as macrophages, B cells, and T cells, which can modulates cell proliferation, cell differentiation, and apoptosis regulation (17). TNF-α is involved in the pathophysiology of bacterial infections by secreting several stimulatory cytokines (18).

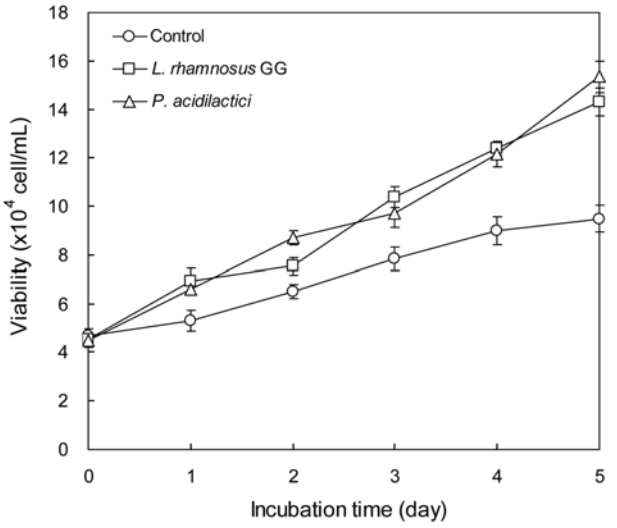

Fig. 2. Effect of the supernatant of B cells cultured with the fermented squid by-products on the proliferation of NK cells.

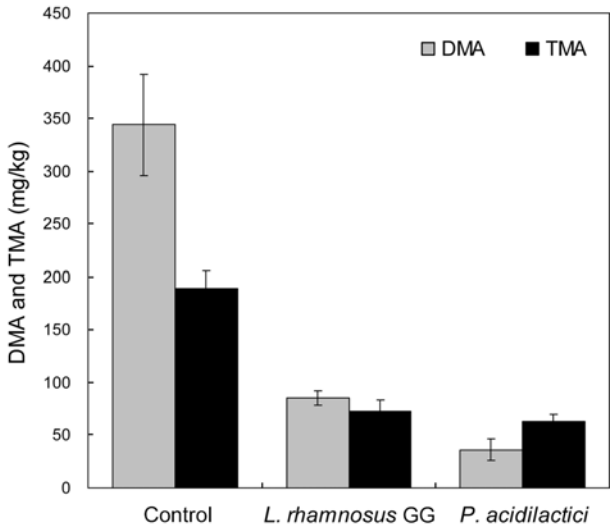

Fig. 3. Reduction of DMA and TMA in squid by-products fermented by different probiotic bacteria for 96 hr at 37°C.

Potential immunomodulating activities of probiotic-fermented squid by-products on the NK cell proliferation Natural killer (NK) cells are considered to be essential cellular players of the immunological processes (19,20). Thus, the NK cells were cultivated in the supernatants of B cells cultured with the squid by-products fermented by selected probiotics. Compared to the control, the soluble fractions promoted significantly ($p<0.05$) the growth of NK cells throughout the cultivation time (Fig. 2). The cytokines, IL-6 and TNF-α, induced from B cells by the selected probiotic bacterial fermented extracts directly regulated NK cell proliferation. The numbers of NK cells in the soluble fractions gained from *L. rhamnosus* and *P. acidilactici* were 1.4×10^5 and 1.5×10^5 cells/mL, respectively, whereas the number of NK cells in the control was 9.5×10^4 cells/mL. *L. rhamnosus*- and *P. acidilactici*-fermented extracts significantly increased the growth of NK cells, which can stimulate the secretion of cytokines. This observation is in good agreement with the reports of Haza *et al*. (21) and Kekkonen *et al*. (22) that probiotic bacteria including *L. sakei*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, and *Bifidobacterium longum* induced the production of cytokines by human macrophages. The induced cytokines may activate the host immune system against tumor cells and microbial infections (23,24).

Changes in DMA and TMA during the fermentation of by-products from squid processing After fermentation, the amounts of DMA and TMA significantly increased as compared to the control (Fig. 3). The amounts of DMA and TMA in *L. rhamnosus*-fermented sample were 85.3 (74% reduction) and 72.4 mg/kg (62% reduction) and those in *P. acidilactici*-fermented sample were 36.1 (89% reduction) and 63.2 mg/kg (66% reduction), respectively. The probiotic bacterial fermentation of squid by-products resulted in a significant reduction of DMA and TMA. The utilization of squid by-products has been limited by TMA and DMA which are formed from the reduction of trimethylamine oxide (TMAO) and responsible for characteristic fishy odor (25,26). Therefore, the results suggest that the selected probiotic bacteria can be used to suppress the production of biogenic amines such as DMA and TMA. Because this is the first study to examine the effect of probiotic bacterial fermentation on the reduction of DMA and TMA, it is worthwhile to provide the information for eliminating the off-flavors.

In conclusion, this study demonstrates that *L. rhamnosus* and *P. acidilactici* are potentially beneficial probiotic strains for the utilization of by-products from squid processing. The significant findings in this study were that the squid processing by-products fermented by 2 selected probiotic strains showed high antioxidant activities, stimulated the cytokine production by B cells, induced a great increase in NK cell proliferation, and decreased DMA and TMA content. Therefore, the probiotic fermentation may be valuable and desirable for utilizing by-products from squid processing.

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