Enhancement of antioxidative and antimicrobial activities of immature pear (*Pyrus pyrifolia* cv. Niitaka) fruits by fermentation with *Leuconostoc mesenteroides*

Sang Won Lee¹, Jeong-Yong Cho¹, Hang Yeon Jeong¹, Tae-Woong Na², Sang-Hyun Lee^{3,4}, and Jae-Hak Moon^{1,*}

¹Department of Food Science and Technology, Functional Food Research Center, and BK21 Plus Program, Chonnam National University, Gwangju 61186, Korea

²Jeonnam Bio Control Center, Gokseong, Jeonnam 57510, Korea

³Korea Pear Research Organization, Chonnam National University, Gwangju 61186, Korea

⁴Department of Horticulture, Chonnam National University, Gwangju 61186, Korea

Received May 16, 2016
Revised August 7, 2016
Accepted October 25, 2016
Published online December 31, 2016

*Corresponding Author
Tel: +82-62-530-2141
Fax: +82-62-530-2149
E-mail: nutrmoon@jnu.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract Immature pear (*Pyrus pyrifolia* cv. Niitaka) fruits were fermented with *Leuconostoc mesenteroides* and *Aspergillus oryzae*, which are commonly used as starters for manufacturing fermented foods. Fermented immature pear fruit extracts (FIPF) by *L. mesenteroides* showed significantly higher radical-scavenging activity using DPPH, ABTS, superoxide anion, and hydroxyl radicals and reducing power capacity than unfermented immature pear fruit extracts. *L. mesenteroides*-FIPF more effectively inhibited the formation of cholesteryl ester hydroperoxide in copper ion-induced rat blood plasma. In addition, the *L. mesenteroides*-FIPF strongly inhibited tyrosinase activity and the growth of pathogenic skin bacteria. In contrast, enhanced antioxidative and antibacterial activities were not apparent in *A. oryzae*-FIPF. The antioxidative and antimicrobial activities of the fermented and unfermented immature pear fruits were correlated with the flavonoid contents. These results indicate that fermentation enhances antioxidative and antimicrobial activities of immature pear fruits.

Keywords: immature pear fruits, *Pyrus pyrifolia*, *Leuconostoc mesenteroides*, antioxidant, antibacterial activity

Introduction

The pear (*Pyrus* spp.) is widely distributed and is one of the most widely consumed fruits in the world (1). The fruit is often used in processed foods such as beverage, puree, jellies, and jams, but the majority is eaten fresh (2). Nevertheless, an investigation into the chemical constituents and biological activities of the pear has not been fully conducted. This might be because pear fruits do not have a characteristic color and flavor, which is different from apple, grape, and citrus fruits. In a series of our recent studies on the chemical constituents contained in pear fruit, various compounds were identified such as phenylpropanoid malate derivatives (3), caffeoyl triterpenes (4), and other phenolics including flavonoids (5–8), from the fruit of *P. pyrifolia* Nakai cv. Chuhwangbae, which is one of the most highly consumed pear cultivars in Korea (3). In addition, the levels of major chemical constituents (arbutin, chlorogenic acid, malaxinic acid, total caffeic acid, total flavonoids, and total phenolics) and antioxidative activities decreased gradually in the fruits of seven pear cultivars as

they developed (9). In other word, phenolic compound contents as well as antioxidative activities in immature pear fruits were overwhelmingly higher than those in mature pear fruits. Therefore, the immature pear fruit might be used as a source of bioactive compounds and functional foods.

Immature pears are handpicked soon after florescence to harvest high quality fruits (10). In this process, one fruit in a cluster of seven or eight often remains and is ultimately discarded (11). Considering the annual output of mature pear fruits (12), the sum of the discarded immature pear fruits is estimated to be about 15,000 tons/year in Korea. Nevertheless, most of the discarded immature pear fruits are unused.

Many studies on fermentation to increase the health benefits of natural food sources have been performed (13,14). To indicate the uses of immature pear fruits, we applied the fermentation method to fruits of *Pyrus pyrifolia* cv. Niitaka, which is the most commonly produced pear fruit in Korea (15). The fermented immature pear fruits (FIPF) were prepared with various food microorganisms,

including seven bacteria and two fungi. The FIPF prepared with *Leuconostoc mesenteroides* (Lm-FIPF) and *Aspergillus oryzae* (Ao-FIPF) showed higher DPPH radical-scavenging activities than those prepared with other microorganisms and these activities were higher than those in the unfermented immature pear fruits (UIPF) (15). In this study, the antioxidative and antimicrobial activities of Lm-FIPF and Ao-FIPF before and after fermentation were compared.

Materials and Methods

Materials Immature pear fruits of *Pyrus pyrifolia* Nakai cv. Niitaka were cultivated in Naju city, Korea. Fresh immature pear fruits were collected on May 15, 2014, 30 days after florescence. The collected immature pear fruits were immediately frozen with dry ice and stored at -70°C . Fermentation of the immature pear fruits was performed within 2 months of storage. The immature pear cultivar used in this study was identified by Prof. Wol-Soo Kim, Laboratory of Pomology, College of Agriculture and Life Science, Chonnam National University. A voucher sample (NTK, JNU PE 20111015) was previously deposited in the herbarium of the laboratory.

Chemicals Gallic acid, (-)-epicatechin, tyrosinase (EC 1.14.18.1, from mushroom), ferulic acid, sodium nitrite, aluminum chloride, ABTS, and benzoic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Miconazole, econazole, DPPH, and copper (II) sulfate were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium carbonate and Folin & Ciocalteu's reagent were purchased from Junsei Chemical Industries Ltd. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals and solvents were of analytical grade.

Microorganisms and culture medium for fermentation *A. oryzae* KCCM 11372 and *L. mesenteroides* KCTC 13374 were obtained from the Korean Culture Center for Microorganisms (KCCM, Seoul, Korea) and the Korean Collection for Type Culture (KCTC, Daejeon, Korea), respectively. The tested microorganisms were grown by subculturing periodically on agar medium and were maintained at 4°C before use. The media used were potato dextrose broth (4 g of potato starch and 20 g of dextrose, per L, pH 5.1) for *A. oryzae* and deMan, Rogosa and Sharpe (MRS) broth (5 g of beef extract, 10 g of dextrose, 2.5 g of sodium acetate, 2.5 g of yeast extract, 5 g of proteose peptone, 1 g of ammonium citrate, 1 g of dipotassium phosphate, 0.5 g of polysorbate 80, and 0.05 g of magnesium sulfate, per L, pH 6.2) for *L. mesenteroides*. The media were sterilized by autoclaving at 15 psi and 121°C . Both microorganisms were incubated for 24 h at 30°C on the base of growth curves (data not shown) and the broths were used for fermentation of immature pear fruits, respectively.

Fermentation of immature pear fruits The immature pear fruits after thawing were homogenized and sterilized. The sterilized

immature pear fruits (50 g fresh wt.) were inoculated with a 3% (v/w) suspension of *A. oryzae* and *L. mesenteroides*, and incubated for 2 days at 30°C and 120 rpm in an incubator.

Extraction of fermented immature pear fruits The UIPF and FIPF (50 g fresh wt.) were homogenized (T-50 Ultra Turrax; Ika, Seoul, Korea) with 150 mL of methanol (MeOH). After standing for 30 min, the homogenates were filtered with filter paper No. 2 (Whatman, Maidenstone, UK). The combined extract solutions were concentrated under vacuum at 38°C . The concentrates (50 g fresh wt. eq./20 mL MeOH) were dissolved with 20 mL of MeOH. These solutions were used to determine the chemical constituents and biological activities.

Determination of total phenolic content Total phenolic content in the fermented immature pear fruits was measured using Folin–Ciocalteu's method (16). Briefly, the MeOH extracts (12.5 mg fresh wt. eq.) were mixed with H_2O (0.5 mL), saturated Na_2CO_3 solution (0.5 mL), and Folin–Ciocalteu phenol reagent (0.5 mL). The mixture was incubated at room temperature for 30 min. Then, the filtrate was analyzed using a spectrophotometer (700 nm, UVmini-1240; Shimadzu, Kyoto, Japan). Total phenolic content (gallic acid equivalent, GAE) of the samples was quantified based on a calibration curve of GA, which was used as the standard.

Determination of total flavonoid content Total flavonoid contents in fermented immature pear fruit extracts were determined using a colorimetric method (9) with slight modifications. Briefly, the MeOH extracts (25 mg fresh wt. eq.) of UIPF and FIPF were mixed with distilled water (900 μL) and 5% sodium nitrate (70 μL), respectively. Each mixture was held at room temperature for 5 min. Then, 10% aluminum chloride solution (150 μL) was mixed with 1 N NaOH (500 μL) and distilled water (280 μL). The mixture was filtered with a 0.45- μm Millipore membrane filter and analyzed using a spectrophotometer (517 nm, Shimadzu). Total flavonoid content was presented in catechin equivalents (CE), based on the standard curve of catechin.

Determination of DPPH radical-scavenging activity Free radical-scavenging activity was determined using DPPH radicals according to the modified method of Cho *et al.* (9). Briefly, the MeOH extracts (37.5 mg fresh wt. eq.) from UIPF and FIPF were added to 1 mL of DPPH radical ethanol solution (500 mM, for a final concentration of 250 mM), respectively. Two milliliters of each solution was completed with ethanol, mixed, and allowed to stand for 30 min in the dark. The absorbance (RS) of the sample mixture was measured at 517 nm. The DPPH radical-scavenging activity of each sample was determined based on the percentage decrease in the absorbance from a blank test. DPPH radical-scavenging activities were determined as follows:

$$\text{DPPH radical-scavenging activity (\%)} = [(\text{Control} - \text{RS}) / \text{Control}] \times 100$$

Determination of ABTS⁺ radical-scavenging activity ABTS⁺ radical-

scavenging activity was determined using the modified method of Dudonne *et al.* (17). Briefly, an ABTS⁺ radical (final concentration 7 mM) was reacted with 2.5 mM potassium persulfate (95:5, v/v) for 12 h at 4°C in the dark. The absorbance of ABTS⁺ solution was adjusted to approximately 0.70±0.15 at 735 nm by dilution with ethanol. The diluted ABTS⁺ radical solution (0.5 mL) was reacted with the MeOH extracts from UIPF and FIPF (1.25 mg fresh wt. eq.) and ferulic acid as a positive control. Each solution was incubated for 30 min in the dark at room temperature, and then centrifuged for 5 min at 9,800×g and 4°C. The absorbance of the supernatant was measured using a spectrophotometer (735 nm, Shimadzu), and the ABTS⁺ radical-scavenging activity was calculated as follows:

$$\text{ABTS}^+ \text{ radical-scavenging activity (\%)} = \frac{[(\text{control} - \text{RS}) / \text{control}]}{1} \times 100$$

Determination of reducing power The reducing powers of UIPF and FIPF were determined using a modification on the method of Oyaizu (18). Briefly, the fermented immature pear fruit extracts (25 mg fresh wt. eq.) were mixed with 80% ethanol (0.5 mL), 0.2 M sodium phosphate buffer (pH 6.6, 0.5 mL), and 1% potassium ferricyanide solution (0.5 mL). Each mixture was incubated in a water bath at 50°C for 20 min. Then, the reacted solution was mixed with 10% trichloroacetic acid solution (0.5 mL). After centrifugation for 5 min at 9,800×g at 4°C, the supernatant (0.75 mL) was diluted with distilled water (0.75 mL). The solution was mixed with 0.1% ferric chloride solution (0.1 mL). The mixture was allowed to stand for 10 min at room temperature and the absorbance was measured at 700 nm. Ferulic acid was used as a positive control.

Determination of hydroxyl radical-scavenging activity Hydroxyl radical-scavenging activity was determined according to the modified method of Xiao *et al.* (13). A mixture of 1 mM FeSO₄ (400 µL), 1 mM phenanthroline (400 µL), and 0.01% H₂O₂ was prepared and added to the MeOH extracts from UIPF and FIPF (100 µL, 2.5 mg fresh wt. eq.). After incubation at 37°C for 20 min, the absorbance of the reaction mixtures was measured at 540 nm. Quercetin was used as a positive control. Hydroxyl radical-scavenging activity was calculated using the following equation:

$$\text{Hydroxyl radical-scavenging activity (\%)} = \frac{[(\text{RS} - \text{control}) / (A_0 - \text{control})]}{1} \times 100,$$

where RS indicates the absorbance of the sample or quercetin with H₂O₂ reagent, and A₀ is the absorbance of the sample or quercetin without H₂O₂ reagent.

Determination of superoxide anion radical-scavenging activity Superoxide anion radical-scavenging activity was determined according to the modified method of Xiao *et al.* (13). A mixture of 390 µM nicotinamide adenine dinucleotide (300 µL) and 250 µM nitroblue tetrazolium (300 µL) was prepared and added to the solution (100 µL) containing the MeOH extracts from UIPF and FIPFs

(20 mg fresh wt. eq.). After addition of 250 µM phenazine methosulfate 100 µL, the mixture was incubated at 37°C for 5 min. The absorbance of the reaction mixture was measured at 540 nm. Ascorbic acid was used as a positive control.

$$\text{Superoxide anion radical-scavenging activity (\%)} = \frac{[(\text{control} - \text{RS}) / \text{control}]}{1} \times 100$$

Determination of the inhibitory effects of UIPF and FIPF against copper ion-induced peroxidation in rat blood plasma

The antioxidative activities of the fermented immature pear fruits were determined by measuring their inhibitory effects against the accumulation of cholesteryl ester hydroperoxide (CE-OOH) with induced oxidation by copper ion in diluted rat blood plasma (19). Rats (Sprague-Dawley, ♂, 6 weeks of age, 180–200 g, Samtako Bio Korea, Osan, Korea) were housed at 23°C under a 12-h dark/light cycle and fasted for 12 h before blood collection. After anesthesia with diethyl ether, the abdomen wall of rat was opened, and blood was collected from the abdominal aorta. The blood containing heparin was centrifuged (1,500×g) at 4°C for 20 min and the isolated plasma was used immediately for experiments or stored at 40°C for no longer than 1 week. The plasma samples (3,000 µL each) were mixed with the MeOH extracts from UIPF and FIPF (6.25 mg fresh wt. eq.) in phosphated buffered saline (PBS, pH 7.4, 650 µL). Oxidation of the mixtures was initiated by the addition of 100 µL CuSO₄-PBS solution (final concentration, 100 µM) and incubated at 37°C for 8 h with continuous shaking. The reaction mixture (100 µL) was mixed with MeOH (3 mL) containing 2.5 mM 2,6-di-tert-butyl-4-methylphenol to obtain CE-OOH. The mixture was sonicated (Power Sonic 4200; Hwashin, Ulsan, Korea) for 1 min, and extracted with *n*-hexane (2 mL, two times) by vortexing vigorously for 1 min to obtain the upper layer (*n*-hexane). Obtained *n*-hexane layers were combined and evaporated in a rotary evaporator at room temperature. The concentrate was dissolved in 100 µL MeOH/CHCl₃ (95:5, v/v), and an aliquot was analyzed by reverse phase-high-performance liquid chromatography (RP-HPLC) using a TSK-gel Octyl-80Ts column (Tosoh, Tokyo, Japan). The mobile phase consisted of MeOH/H₂O (97:3, v/v), and was eluted in 1.0 mL/min of flow rate. The CE-OOH was analyzed at 235 nm using UV detector (SPD-10A; Shimadzu). The concentration of CE-OOH was determined from a standard curve of cholesteryl linoleate hydroperoxide.

Determination of tyrosinase inhibitory activity Tyrosinase inhibitory activity was measured according to the modified method of Gurme *et al.* (20). The MeOH extracts of UIPF and FIPF (100 µL, 75 mg fresh wt. eq.) were mixed each with 40 µL of 2 mM L-3,4-dihydroxy-phenylalanine (DOPA), 80 µL of 67 mM sodium phosphate buffer (pH 6.8), and 40 µL of tyrosinase (125 unit/mL). After incubation at 37°C for 30 min, the mixture was partitioned with ethyl acetate (200 µL, 2 times). The H₂O layer was subjected to RP-HPLC with a Capcell Pak C18 AQ (4.6×250 mm, Shiseido, Tokyo, Japan). The

sample was eluted with H₂O containing 2% acetic acid as the mobile phase at a flow rate of 1.0 mL/min with monitoring at 280 nm (Shimadzu). Tyrosinase inhibitory activity of each sample was reversely calculated with decreasing rate of DOPA peak area from a blank test on HPLC chromatogram.

Determination of antimicrobial activity Five pathogenic microorganisms (*Escherichia coli* KCTC 1924, *Pseudomonas aeruginosa* KCTC 2004, *Staphylococcus aureus* KCTC 1927, *Staphylococcus epidermidis* KCTC 1917, and *Candida albicans* KCTC 2959) were obtained from KCTC. All microorganisms tested were periodically grown on agar medium and were held at 4°C before use. The media used to test the microorganisms were Yeast Malt Broth (Difco) for *C. albicans*, Luria Bertani Broth (Difco) for *E. coli* and *P. aeruginosa*, and Nutrient Broth (Difco) for *S. aureus* and *S. epidermidis*. The incubation temperature for all bacteria except for *C. albicans* (25°C) was to 37°C. The media were sterilized by autoclaving at 15 psi and 121°C. All five microorganisms were cultured in their respective broth media before use.

The antimicrobial activities of UIPF and FIPF were measured by an agar disc diffusion method (21). Sterilized agar (80 mL) containing a cultured microorganism (0.2 mL) was extended with equal thickness on petriplates (ϕ 15 cm petri dish, Corning, New York, USA). The MeOH extracts from UIPF and FIPF (250 mg fresh wt. eq.) were dissolved in MeOH (25 μ L), then used to impregnate sterilized paper discs (diameter 6 mm, Advantec Toyo Kaisha Ltd., Tokyo, Japan). A paper disc treated with a MeOH extract from pear fruit was placed on a solid plate. Each microorganism was incubated at the optimal growth temperature for 16 h and the antimicrobial activity was measured by the diameter (mm) of inhibition zone. Miconazole (225 nmol), econazole (225 nmol), and benzoic acid (9 μ mol) were used as positive controls.

Statistical analysis Data were displayed as the mean $\pm$ standard deviation (SD) using the Statistical Package for Social Sciences 19.0 program (SPSS Inc., Chicago, IL, USA). Statistical differences were assessed by one-way analysis of variance followed by the Turkey-Kramer test. Values of $p < 0.05$ were considered significant.

Result and Discussion

Total phenolic and flavonoid contents Total flavonoid and phenolic contents in the MeOH extracts of Lm- and Ao-FIPFs were determined by the colorimetric method with aluminum chloride (9) and Folin-Ciocalteu's method (16), respectively. The total flavonoid and phenolic contents of Lm- and Ao-FIPFs are shown in Fig. 1. Lm-FIPF showed significantly higher total flavonoid content (85.6 $\pm$ 4.4 mg CE/100 g fresh wt. eq.) than Ao-FIPF (54.7 $\pm$ 2.1 mg/100 g fresh wt. eq.) or UIPF (55.4 $\pm$ 2.4 mg CE/100 g fresh wt. eq.) (Fig. 1A). There was no significant difference in total flavonoid content between Ao-FIPF and

Fig. 1. Total flavonoid (A) and phenolic (B) contents of fermented immature pear fruits. UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Different letters indicate significant differences at $p < 0.05$.

UIPF. The total phenolic contents of Lm- and Ao-FIPFs were 130.7 $\pm$ 2.3 and 141.8 $\pm$ 7.8 mg GAE/100 g fresh wt. eq., respectively, which were significantly higher than the content of UIPF (119.3 $\pm$ 3.4 mg GAE/100 g fresh wt. eq.) (Fig. 1B). In addition, total phenolic content in Ao-FIPF was significantly ($p < 0.05$) higher than that in UIPF. However, there was no significant difference in total phenolic content between Lm-FIPF and UIPF.

DPPH radical-scavenging activity The radical-scavenging activities of Lm- and Ao-FIPF MeOH extracts were evaluated using DPPH radicals. The UIPF and FIPF (MeOH extract, 37.5 mg fresh wt. eq.) showed significantly higher DPPH radical-scavenging activity than α -tocopherol activity (39.2 $\pm$ 1.5% at a concentration of 50 μ g), which was used as a positive control (Fig. 2A). Interestingly, Lm-FIPF (94.1 $\pm$ 0.1%) exhibited higher DPPH radical-scavenging activity than UIPF (75.3 $\pm$ 0.3%) (Fig. 2A). In contrast, Ao-FIPF (69.5 $\pm$ 0.4%) showed weaker DPPH radical-scavenging activity than UIPF.

ABTS⁺ radical-scavenging activity The radical-scavenging activities of the Lm- and Ao-FIPF MeOH extracts were evaluated using ABTS⁺ radicals. Lm-FIPF (79.6 $\pm$ 1.0%) (MeOH extract, 37.5 mg fresh wt. eq.) showed higher ABTS⁺ radical-scavenging activity than either UIPF

Fig. 2. DPPH (A) and ABTS⁺ (B) radical-scavenging activities and reducing power (C) of the fermented immature pear fruits. UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Different letters indicate significant differences at $p < 0.05$.

(54.5 $\pm$ 1.5%) or Ao-FIPF (57.9 $\pm$ 0.3%) (Fig. 3B). However, the ABTS⁺ radical-scavenging activities of UIPF and Ao-FIPF were not significantly different ($p < 0.05$) (Fig. 2B). These results were similar to those of DPPH radical-scavenging activity, described above.

Reducing power The reducing powers of UIPF and FIPF are shown in Fig. 2C. The reducing power of Lm-FIPF was significantly higher than that of UIPF. In addition, the Lm-FIPF MeOH extracts (0.52 $\pm$ 0.03, absorbance values at 700 nm) at a concentration of 25 mg fresh wt. eq. showed significantly higher reducing capability than ferulic acid (50 μ g, 0.222 $\pm$ 0.032, absorbance values at 700 nm). However, Ao-

Fig. 3. Hydroxyl (A) and superoxide anion (B) radical-scavenging activities of fermented immature pear fruits. UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Different letters indicate significant differences at $p < 0.05$.

FIPF (0.132 $\pm$ 0.002, absorbance values at 700 nm) at a concentration of 25 mg fresh wt. eq. was similar to UIPF (0.132 $\pm$ 0.002, absorbance values at 700 nm). These capacities were lower than that (50 μ g, 0.222 $\pm$ 0.032, absorbance values at 700 nm) of ferulic acid.

Hydroxyl radical-scavenging activity The hydroxyl radical-scavenging activities of UIPF and FIPF are shown in Fig. 3A. Lm-FIPF (MeOH extract, 2.5 mg fresh wt. eq.) showed significantly higher hydroxyl radical-scavenging activity (87.4 $\pm$ 2.9%) than either UIPF (59.8 $\pm$ 7.6%) or Ao-FIPF (63.5 $\pm$ 2.9%). In addition, Lm-FIPF was more effective in higher hydroxyl radical-scavenging than quercetin (67.9 $\pm$ 3.8%) at a concentration of 2 μ g. However, the hydroxyl radical-scavenging activities of UIPF and Ao-FIPF were not significantly different ($p < 0.05$).

Superoxide anion radical-scavenging activity The superoxide anion radical-scavenging activities of UIPF and FIPF are shown in Fig. 3B. UIPF and FIPF (2.5 mg fresh wt. eq.) showed significantly higher superoxide anion radical-scavenging activity than that of ascorbic acid at a concentration of 10 μ g (Fig. 3B). Lm-FIPF showed the highest superoxide anion radical-scavenging activity (69.3 $\pm$ 0.4%). However, Ao-FIPF (55.1 $\pm$ 1.0%) exhibited significantly lower superoxide

Fig. 4. Inhibition effect of fermented immature pear fruits against CE-OOH formation resulting from copper ion-induced oxidation in rat blood plasma. UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Different letters indicate significant differences at $p < 0.05$.

anion radical-scavenging activity than UIPF ($64.9 \pm 2.7\%$).

Inhibitory activity against CE-OOH formation in rat blood plasma

After the addition of the MeOH extracts (6.25 mg fresh wt. eq.) of UIPF and FIPF in plasma, lipid peroxidation was initiated by adding copper ions, and the inhibitory effect of the pear fruit extracts was determined by measuring the CE-OOH concentration (Fig. 4). The CE-OOH concentration in copper ion-induced rat blood plasma of the control group was $21.8 \pm 1.1 \mu\text{M}$. Interestingly, UIPF, Lm-FIPF, and Ao-FIPF all strongly inhibited CE-OOH formation in the copper-induced rat blood plasma. In particular, Lm-FIPF ($5.9 \pm 0.6 \mu\text{M}$) exhibited a significantly stronger inhibitory effect against CE-OOH formation than UIPF ($9.0 \pm 0.1 \mu\text{M}$) and Ao-FIPF ($10.1 \pm 0.3 \mu\text{M}$). Ao-FIPF showed slightly greater inhibition of CE-OOH formation than UIPF, but this difference was not significant.

Tyrosinase inhibitory activity The tyrosinase inhibitory activities of UIPF and FIPF are shown in Fig. 5. At a concentration of 75 mg fresh wt. eq., tyrosinase inhibitory activity was as follows: Lm-FIPF ($64.3 \pm 6.6\%$) > UIPF ($35.3 \pm 9.6\%$) > Ao-FIPF ($24.8 \pm 5.1\%$). The tyrosinase inhibitory activity of Lm-FIPF was slightly higher than that of ascorbic acid at a

Fig. 5. Tyrosinase inhibitory activity of the fermented immature pear fruits. UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Different letters indicate significant differences at $p < 0.05$.

concentration of $10 \mu\text{g}$, although this difference was not significant. However, Ao-FIPF exhibited lower tyrosinase inhibitory activity than UIPF.

Antimicrobial activity The antimicrobial activities of UIPF and FIPF against food spoilage and pathogenic skin microorganisms were evaluated by an agar disc diffusion method. The UIPF and FIPF strongly inhibited the growth of four different microorganisms, but not the growth of *C. albicans* (Table 1). In particular, the UIPF and FIPF were more effective against *S. aureus* and *S. epidermidis* when compared to the other tested microorganisms. Lm-FIPF showed higher antibacterial activity than UIPF and Ao-FIPF. Although Ao-FIPF showed weaker antimicrobial activity than UIPF, the inhibitory activities were still similar to benzoic acid at the concentration ($9 \mu\text{mol}$) against all the tested microorganisms, except for *C. albicans*. In contrast, UIPF and FIPF did not inhibit the growth of *C. albicans*, even though higher activities of miconazole, econazole, tetracycline, and benzoic acid, which have commonly been used to eradicate skin pathogenic microorganisms, were observed.

Oxidative damage causes various human diseases including cardiovascular diseases, atherosclerosis, cancer, hypertension, diabetes,

Table 1. Antimicrobial activities of fermented immature pear fruits against pathogenic bacteria

Strains	Inhibition zone (mm)			
	UIPF	Lm-FIPF	Ao-FIPF	Benzoic acid
<i>Escherichia coli</i>	7.7 ± 0.6^a	9.3 ± 0.6^b	7.3 ± 0.6^a	7.3 ± 0.6^a
<i>Pseudomonas aeruginosa</i>	9.3 ± 0.6^b	11.3 ± 0.6^c	7.0 ± 0.0^a	6.7 ± 0.6^a
<i>Staphylococcus aureus</i>	11.7 ± 0.6^b	14.3 ± 0.6^c	10.3 ± 0.6^a	11.3 ± 0.6^{ab}
<i>Staphylococcus epidermidis</i>	12.3 ± 0.6^b	13.7 ± 0.6^c	10.0 ± 0.0^a	10.3 ± 0.6^a
<i>Candida albicans</i>	-	-	-	17.3 ± 0.6

UIPF, unfermented immature pear fruits; Lm-FIPF, *L. mesenteroides*-fermented immature pear fruits; Ao-FIPF, *A. oryzae*-fermented immature pear fruits. Benzoic acid was used as a positive control. Miconazole ($21.0 \pm 1.0 \text{ mm}$) and econazole ($21.0 \pm 1.0 \text{ mm}$) showed inhibitory activity against *C. albicans*, but did not inhibit the growth of the other bacteria. -: No growth inhibition. Values represent mean $\pm$ standard deviation ($n=3$). Different letters in the same row indicated a significant difference ($p < 0.05$) by the Tukey-Kramer test.

as well as aging (22,23). Oxidative damage is a result of excessive reactive oxygen species and free radicals in the body, and it is well known that antioxidant-rich fruits reduce the risk of cardiovascular disease and cancer via attenuation of this damage (24). Fruits are a useful source of vitamins and phytochemicals, including antioxidants. In particular, phenolics such as flavonoids and phenolic acids in fruits have received considerable attention as antioxidants (25). The results of this study showed that the FIPF had higher total phenolic and flavonoid contents than UIPF. In particular, a significantly higher total phenolic content in Ao-FIPF and a higher total flavonoid content in Lo-FIPF were observed when compared to that of UIPF (Fig. 1). Phenolic compounds, including flavonoids, are widely present as glycosides in fruits (26). Several reports have shown that *A. oryzae* and *L. mesenteroides* exert β -glycosidase activity (27,28). Therefore, it can be inferred that the free form of aglycones of phenolic compounds, including flavonoids, are produced by the glycosidases secreted from *A. oryzae* and *L. mesenteroides* during fermentation. We found that Lm-FIPF had a significantly higher total flavonoid content than Ao-FIPF, although the total phenolic contents were not significantly different. This result suggests that *L. mesenteroides* secretes specific glycosidases that hydrolyze flavonoid glycosides.

The free radical-scavenging activities of UIPF and FIPF were measured using DPPH, ABTS^{•+}, hydroxyl, and superoxide radicals. The results of these *in vitro* tests showed that Lm-FIPF exerted greater free radical-scavenging activity than UIPF (Fig. 2 and 3). However, Ao-FIPF showed lower free radical-scavenging activities than UIPF. Interestingly, the free radical-scavenging and reducing capacities of UIPF and FIPF were correlated with their flavonoid contents. However, antioxidative activity did not correlate with total phenolic content in UIPF. These results suggest that the free radical-scavenging activities of UIPF may have been contributed mainly by flavonoid aglycones produced from their glycosides during fermentation.

CE-OOH is present in healthy human blood plasma at a concentration of approximately 3 nM (29). The CE-OOH overproduced from oxidation accumulates in atherosclerotic plaques as lesion progress (30). Therefore, CE-OOH has been used as a blood plasma lipid peroxidation index (31). In this study, we showed that Lm-FIPF more effectively inhibited CE-OOH formation during lipid peroxidation in rat plasma than both UIPF and Ao-FIPF (Fig. 4). It indicates that the fermented immature pear fruits produced with *L. mesenteroides* have high inhibitory effect against CE-OOH formation associated with lipid peroxidation of rat blood plasma induced by copper ions.

Arbutin strongly inhibits mammalian and mouse melanoma tyrosinases (32) and has been widely used as a whitening agent in cosmetic products. This compound is one of the most abundant compounds in pear fruit. Therefore, the tyrosinase inhibitory activities of the UIPF and FIPF were evaluated using mushroom tyrosinase. Lm-FIPF showed higher tyrosinase inhibitory activity, while Ao-FIPF showed lower activity, compared to UIPF (Fig. 5). The tyrosinase inhibitory activities of UIPF and FIPF were proportionally correlated with their flavonoid contents. Flavonoids such as

kaempferol and quercetin have been reported to strongly inhibit tyrosinase (33). In our previous studies, immature pear fruits showed high total flavonoid contents, and various flavonoids have been identified from the fruits (5,9). Therefore, Lm-FIPF having high total flavonoid content might effectively inhibit tyrosinase.

Infection of skin by pathogenic bacteria is the major health concern in the world. Skin and wound infections are frequently caused by pathogenic bacteria such as *E. coli*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *C. albicans* (34). In this study, we found that UIPF and FIPFs showed antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*, but not against *C. albicans* by the agar diffusion assay. Interestingly, Lm-FIPT exhibited the greatest inhibition on the growth of the four bacteria. The antibacterial activities of UIPF and FIPF correlated with their flavonoid contents, but not with their total phenolic contents. Flavonoids has been reported to have antibacterial activity via inhibition of DNA gyrase, cytoplasmic membrane function, and energy metabolism (35). Therefore, the flavonoid aglycones of fermented immature pear fruits may be at least partially attributed to inhibition of the growth of bacteria via various mechanisms.

Lm-FIPF showed higher radical-scavenging activities, reducing power capacity, and inhibitory effect against CE-OOH formation than UIPF. Moreover, Lm-FIPF strongly inhibited tyrosinase activity and the growth of pathogenic skin bacteria, such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*. The antioxidative and antimicrobial activities of the immature pear fruits may be attributed to flavonoid aglycones produced from their glycosides by fermentation. Therefore, *L. mesenteroides* is a fermenting microorganism that may be used to enhance antioxidative and antibacterial activities of immature pear fruits. Moreover, the fermented immature pear fruits prepared with *L. mesenteroides* can be used to prevent oxidative degradation in foods and to inhibit melanin synthesis and the growth of pathogenic skin bacteria in cosmetic products. Therefore, this study opens possibilities for the use of immature pear fruits, which have mostly been discarded, to improve human health.

Acknowledgment This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Disclosure The authors declare no conflict of interest.

References

1. Lin LZ, Harnly JM. Phenolic compounds and chromatographic profiles of pear skins (*Pyrus* spp.). *J. Agr. Food. Chem.* 56: 9094-9101 (2008)
2. Tanrioven D, Eksi A. Phenolic compounds in pear juice from different cultivars. *Food Chem.* 93: 89-93 (2005)
3. Lee KH, Cho JY, Lee HJ, Ma YK, Kwon J, Park SH, Lee SH, Cho JA, Kim WS, Park KH, Moon JH. Hydroxycinnamoylmalic acids and their methyl esters from pear (*Pyrus pyrifolia* Nakai) fruit peel. *J. Agr. Food Chem.* 59: 10124-10128 (2011)
4. Cho JY, Kim CH, Lee HJ, Lee SH, Cho JA, Kim WS, Park KH, Moon JH. Caffeoyl triterpenes from pear (*Pyrus pyrifolia* Nakai) fruit peels and their antioxidative

- activities against oxidation of rat blood plasma. *J. Agr. Food Chem.* 64: 4563-4569 (2013)
5. Lee YG, Cho JY, Kim CM, Lee SH, Kim WS, Jeon TI, Park KH, Moon JH. Coumaroyl quinic acid derivatives and flavonoids from immature pear (*Pyrus pyrifolia* Nakai) fruit. *Food Sci. Biotechnol.* 22: 803-810 (2013)
 6. Lee KH, Cho JY, Lee HJ, Park KY, Ma YK, Lee SH, Cho JA, Kim WS, Park KH, Moon JH. Isolation and identification of phenolic compounds from an Asian pear (*Pyrus pyrifolia* Nakai) fruit peel. *Food Sci. Biotechnol.* 20: 1539-1545 (2011)
 7. Cho JY, Lee YG, Lee SH, Kim WS, Park KH, Moon JH. An ether and three ester derivatives of phenylpropanoid from pear (*Pyrus pyrifolia* Nakai cv. Chuhwangbae) fruit and their radical-scavenging activity. *Food Sci. Biotechnol.* 23: 253-259 (2014)
 8. Lee YG, Cho JY, Kim CM, Jeong HY, Lee DI, Kim SR, Lee SH, Kim WS, Park KH, Moon JH. Isolation and identification of 3 low-molecular compounds from pear (*Pyrus pyrifolia* Nakai cv. Chuhwangbae) fruit peel. *Korean J. Food Sci. Technol.* 45: 174-179 (2013)
 9. Cho JY, Kim EH, Yun HR, Jeong HY, Lee YG, Kim WS, Moon JH. Change in chemical constituents and free radical-scavenging activity during pear (*Pyrus pyrifolia*) cultivar fruit development. *Biosci. Biotech. Bioch.* 79: 260-270 (2015)
 10. Lee YG, Cho JY, Park J, Lee SH, Kim WS, Park KH, Moon JH. Large-scale isolation of highly pure malaxinic acid from immature pear (*Pyrus pyrifolia* Nakai) fruit. *Food Sci. Biotechnol.* 22: 1539-1545 (2013)
 11. Lee SW, Yu Geon Lee YG, Cho JY, Kim YC, Lee SH, Kim WS, Moon JH. Establishment of a simple method for purification of high purity chlorogenic acid from immature fruit of pear (*Pyrus pyrifolia* Nakai). *Appl. Biol. Chem.* 58: 335-341 (2015)
 12. Cho JY, Park KY, Lee KH, Lee HJ, Lee SH, Cho JA, Kim WS, Shin SC, Park KH, Moon JH. Recovery of arbutin in high purity from fruit peels of pear (*Pyrus pyrifolia* Nakai). *Food Sci. Biotechnol.* 20: 801-807 (2011)
 13. Xiao Y, Wang L, Rui X, Li W, Chen X, Jiang M, Dong M. Enhancement of the antioxidant capacity of soy whey by fermentation with *Lactobacillus plantarum* B1-6. *J. Funct. Foods* 12: 33-44 (2015)
 14. Wang YC, Yu RC, Chou CC. Antioxidant activities of soy milk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiol.* 23: 128-135 (2006)
 15. Lee SW. Study on availability of immature pear fruits: Bioactivity of fermented product and large-scale purification of arbutin. MS thesis, Chonnam National University, Gwangju, Korea (2015)
 16. Singleton VL, Rossi JA, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* 16: 144-158 (1965)
 17. Dudonne S, Virtac X, Coutiere P, Woillez M, Merillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD and ORAC assays. *J. Agr. Food. Chem.* 57: 1768-1774 (2009)
 18. Oyaizu M. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* 44: 307-315 (1986)
 19. Kim JY, Cho JY, Ma YK, Lee YG, Moon JH. Nonallergenic urushiol derivatives inhibit the oxidation of unilamellar vesicles and of rat plasma induced by various radical generators. *Free Radical Bio. Med.* 71: 379-389 (2014)
 20. Gurme ST, Surwase SN, Patil SA, Jadhav JP. Evaluation of various factors affecting bioconversion of L-tyrosine to L-DOPA by yeast *Yarrowia lipolytica*-NCIM 3450 using response surface methodology. *Nat. Prod. Bioprospect.* 4: 141-147 (2014)
 21. Zaika LZ. Spices and herbs. Their antimicrobial activity and its determination. *J. Food Safety* 9: 97-118 (1998)
 22. Slavin JL, Lloyd B. Health benefits of fruits and vegetables. *Adv. Nutr.* 3: 506-516 (2012)
 23. Boeing H, Bechthold A, Bub A, Ellinger S, Haller D, Kroke A, Leschik-Bonner E, Muller M, Oberritter H, Achulze M, Stehle P, Watzl B. Critical review: Vegetables and fruit in the prevention of chronic diseases. *Eur. J. Nutr.* 51: 637-663 (2012)
 24. Prior RL. Fruits and vegetables in the prevention of cellular oxidative damage. *Am. J. Clin. Nutr.* 78: 570-578 (2003)
 25. Seifried HE, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.* 11: 567-579 (2007)
 26. Merken HM, Beecher GR. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agr. Food Chem.* 48: 577-599 (2000)
 27. Lee YG, Cho JY, Kim YM, Moon JH. Change in flavonoid composition and antioxidative activity during fermentation of onion (*Allium cepa* L.) by *Leuconostoc mesenteroides* with different salt concentrations. *J. Food Sci.* 81: C1385-C1393 (2016)
 28. Langston J, Sheehy N, Xu F. Substrate specificity of *Aspergillus oryzae* family 3 β -glucosidase. *Biochim. Biophys. Acta* 1764: 972-978 (2006)
 29. Leitinger, N. Cholesteryl ester oxidation products in atherosclerosis. *Mol. Aspects Med.* 24: 239-250 (2003)
 30. Upston JM, Niu X, Brown AJ, Mashima R, Wang H, Senthilmohan R, Kettle AJ, Dean RT, Stocker R. Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. *Am. J. Pathol.* 160: 701-710 (2002)
 31. Yamamoto Y, Niki E. Presence of cholesteryl ester hydroperoxides in human blood plasma. *Biochem. Biophys. Res. Co.* 165: 988-993 (1989)
 32. Funayama M, Arakawa H, Yamamoto R, Nishino T, Shin T, Murao S. Effect of α - and β -arbutin on activity of tyrosinases from mushroom and mouse melanoma. *Biosci. Biotech. Bioch.* 59: 143-144 (1995)
 33. Orhan IE, Khan MT. Flavonoid derivatives as potent tyrosinase inhibitors - a survey of recent findings between 2008-2013. *Curr. Top. Med. Chem.* 14: 1486-1493 (2014)
 34. Cogen AL, Nizet V, Gallo RL. Skin microbiota: A source of disease or defence? *Brit. J. Dermatol.* 158: 442-455 (2008)
 35. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Ag.* 26: 343-356 (2005)