



## Rice bran fermentation by lactic acid bacteria to enhance antioxidant activities and increase the ferulic acid, $\rho$ -coumaric acid, and $\gamma$ -oryzanol content

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Received: 19 July 2019 / Accepted: 5 August 2019 / Published Online: 30 September 2019  
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**Abstract** Rice bran is considered a natural source of antioxidants. In this study, rice bran was fermented with lactic acid bacteria to increase its antioxidant activity. Four strains isolated from fermented food, *Lactobacillus plantarum* MJM60383, *Lactococcus lactis* subsp. *lactis* MJM60392, *Lactobacillus fermentum* MJM60393, and *Lactobacillus paracasei* MJM60396, were confirmed as safe through stability tests such as safety assessment for biogenic amine production, hemolytic activity, and mucin degradation, and showed high reducing capacity. The antioxidant activity of rice bran fermentation altered by these strains was evaluated using several methods including measurement of  $\text{Fe}^{2+}$  chelating activity and scavenging activity by 1,1-diphenyl-2-picryl-hydrazil (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and nitric oxide assays. In this study, the total phenolic content and  $\gamma$ -oryzanol were evaluated by high-performance liquid chromatography. Compared to non-fermented rice bran and a commercial product, rice bran fermented with *Lactococcus lactis* subsp. *lactis* MJM60392 showed the highest phenolic content (844.13 mg GAE/g). Moreover, the content of ferulic acids,  $\rho$ -coumaric acid, and  $\gamma$ -oryzanol in rice bran increased after fermentation with *L. lactis* subsp. *lactis* MJM60392 and *L.*

*fermentum* MJM60393 compared to other samples. Indeed, the DPPH radical scavenging activity and NO scavenging activity were also found to be high in these fermented rice brans. These results indicated that fermentation with lactic acid bacteria increases the active compound levels and the potent antioxidant activities of rice bran.

**Keywords** Antioxidant · *Lactobacillus* · Phenolic · Rice bran

### Introduction

Plants naturally synthesize medicinal compounds such as phytochemicals, which are biologically active compounds present in the roots and leaves of plants [1]. Phytochemicals have been used since a long time to prevent and treat various human diseases [2], and are divided into three major groups: terpenoids, phenolics, and alkaloids [3]. According to numerous studies, cancer risk is lowered when people consume large amounts of fruits and vegetables [4]. Therefore, consumption of plant-based foods may help prevent cancer, increase antioxidant activity, reduce blood cholesterol, lower inflammatory activity, and inhibit cholesterol oxidation [5,6].

Rice bran is a by-product of the rice milling process and includes the pericarp, testa, nucleus, aleurone layer, and embryo of the rice grain. It contains approximately 95% of the nutrients in rice, with the aleurone layer containing high levels of bioactive compounds [7]. Rice bran has many beneficial health effects such as anti-carcinogenic, cholesterol-lowering, anti-hypertensive, anti-cancer, anti-allergy, and antioxidant activities [8-10]. Rice bran contains natural antioxidant compounds such as  $\gamma$ -oryzanol (0.9-2.9%), vitamin E (0.1-0.14%) compounds such as tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) and tocotrienols ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), carotenoids, and phenolic acids [11]. Although rice bran contains many natural antioxidant

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components and nutritional proteins, its potential application as a natural material for preparing functional foods is limited because of its high insolubility, high fiber content, and possible hull contamination [12,13]. The main phenolic compounds in the rice bran layer are phenyl propanoids, which include hydroxycinnamic acids (gallic acid, vanillic acid, hydroxybenzoic acid, and syringic acid) and hydroxybenzoic acid (caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, and sinapic acid) derivatives [14]. These compounds are present as soluble free phenolic acids and insoluble bound phenolic acids. The human body cannot digest the insoluble bound phenolic acids.

Fermentation using lactic acid bacteria (LAB) can hydrolyze the insoluble bound phenolic acids to soluble phenolic acids through the activities of various enzymes such as cellulase, esterase, and decarboxylase to hydrate the insoluble bound phenolic acids [15,9]. The levels of nutrients such as folates, soluble dietary fiber, and total content of phenolic compounds in cereals can be increased by cereal-based LAB fermentation [16]. Many studies have shown that fermentation using probiotics can increase the content of active compounds and antioxidant compounds included in rice bran [17,18]. Thus, many companies have introduced various fermented rice bran products to the market.

However, though the currently available fermented rice bran products are healthy, they are not satisfactory enough in terms of functional and nutritional properties. The purpose of this study was to develop a novel functional rice bran product fermented with different LAB. After the fermentation process, the phenolic acid and hydroxybenzoic acid content was determined. Moreover, rice bran fermented with the selected LAB strains was investigated to determine the antioxidant activities and was compared to a commercial rice bran product A to evaluate its potential for application as a functional food.

## Materials and Methods

### Screening for safety assessment of LAB

All 150 LAB strains used in the study were previously isolated from traditional fermented foods (including yogurt, kefir, cheese, kimchi, and jeotgal) and were obtained from the culture collection of the microbiology laboratory of Chonnam National University. Hemolytic activity was determined as described by Taixera (2014). Briefly, LAB strains were streaked onto Columbia agar plates (Difco, Detroit, MI, USA) containing 5% (vol/vol) defibrinated sheep blood (Medexx Co., Ltd., Gyeonggi-do, Korea). After incubation at 37 °C for 24 h, negative hemolysis activity was observed as the absence of a clear zone around colonies. The biogenic amine method was carried out as described by Bover-Cid, Holzapfel [19]. The LAB strains were incubated in decarboxylase agar (Difco) with or without amino acids at 37 °C for 24 h. Positive activity was indicated by the formation of a purple halo around a colony. Mucinolytic activity was assessed as

described by Le, Yang [20]. All experiments were performed at least three times. Only 27 LAB strains were selected as safe for human consumption and were evaluated further to determine their reducing power capacities.

### Screening for antioxidant activity by reducing power capacity

The reducing activity of rice bran extracts was analyzed as described by Oyaizu [21]. An overnight LAB culture was adjusted to OD 600 nm of 0.5 followed by centrifugation at 3,000×g for 20 min. The cell-free supernatant was then mixed with 1% potassium ferricyanide solution. The mixture was incubated with 10% trichloroacetic acid (w/v) at 50 °C for 25 min. The upper layer was harvested by centrifugation at 150×g for 10 min and mixed with 5 mL deionized water and 1 mL of 0.1% ferric chloride. Finally, the OD 700 nm of the upper layer was measured after 20 min and ascorbic acid was used as a positive control.

### Preparation of rice bran fermentation

Four LAB isolates, *Lactobacillus plantarum* MJM60383, *Lactococcus lactis* subsp. *lactis* MJM60392, *Lactobacillus fermentum* MJM60393, and *Lactobacillus paracasei* MJM60396, were grown on MRS agar plates and subcultured in MRS broth at 37 °C for 24 h under aerobic conditions before fermentation. The rice bran used in this study was purchased from Inno-Nutribio (Seoul, Korea). A milled rice bran sample was passed through a 250-μm sieve. Rice bran (100 g) was then homogenized with 25 mL nutrient solution (2 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1 g L<sup>-1</sup> MgSO<sub>4</sub>, 8 g L<sup>-1</sup> NH<sub>2</sub>CONH<sub>2</sub> in 0.4 N HCl) in tray bioreactors. The reactors were heated at 121 °C for 15 min and then cooled to room temperature. The inoculum of the four LAB strains was added separately and incubated at 37 °C in a fermentation chamber with controlled humidity. After 24 h of fermentation, the rice bran residues were extracted, and then their antioxidant activity and bioactive compound content were determined.

### Fermented rice bran extraction

Fermented rice bran was extracted as described by Lister, Wilson [22]. Samples of 5 g were subjected to orbital shaking (150 rpm) at room temperature for 3 h with methanol and the obtained extract was filtered through filter paper (Whatman no. 4) into a separating funnel. The methanolic extract was evaporated on a rota-evaporator at 50 °C under reduced pressure and was then resuspended in 10 mL of distilled water followed by placing in an ultrasonic bath for 10 min. The resulting extract was clarified with 5 mL of 0.1 M ZnSO<sub>4</sub> and 5 mL of 0.1 M Ba(OH)<sub>2</sub>, and was allowed to rest for 20 min. After centrifugation (10 min, 25 °C, 3,200×g), the supernatant containing the phenolic compounds was collected, lyophilized, and quantified spectrophotometrically at 750 nm with Folin-Ciocalteu reagent (Qell, Brazil) using gallic acid (Sigma, St. Louis, MO, USA) as a standard (2–20 g/mL). Each sample extract was diluted with 25% methanol for antioxidant activity measurement.

### Analysis of phenolic acids and $\gamma$ -oryzanol content

All methanolic extracts were filtered through a 0.25- $\mu$ m syringe filter before use. Phenolic acid compounds were quantified by high-performance liquid chromatography (HPLC; Series 1200, Agilent Technologies, Santa Clara, CA, USA) coupled with a C-18 column (250 nm $\times$ 4.6 mm i.d., 5  $\mu$ m, YMC-Pack ODS AM (YMC Co. Ltd., Kyoto, Japan). The mobile phase was 100% methanol (solvent A) and distilled water with 0.2% glacial acetic acid (solvent B), flow rate was 1 mL/min, and injection volume was 20  $\mu$ L. The mobile phase used to evaluate  $\gamma$ -oryzanol was methanol/acetonitrile/acetic acid (52:45:3 v/v) and the flow rate was set to 0.8 mL/min. The eluent band of phenolic acids (*p*-coumaric acid and ferulic acid) was detected at a wavelength of 325 nm, whereas  $\gamma$ -oryzanol was detected at 325 nm. The results were compared to those obtained using a non-fermented (NA) sample and commercial product A. As stated on the label claim, the composition of commercial product A is as follows: germinated brown rice (30%), rice bran (65%), four kinds of LAB (4.7%), and biominerals (0.3%).

### Determination of antioxidant activity

The ABTS free radical scavenging activity assay was performed as described previously [23]. First, 7 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution was mixed and incubated with 2.4 mM potassium persulfate for 16 h at room temperature in the dark. The mixture was diluted by mixing 1 mL ABTS solution with distilled water to obtain an absorbance of  $0.70 \pm 0.02$  units at 734 nm measured using a UV/Vis spectrophotometer. Sample extracts (10  $\mu$ L) were reacted with 190  $\mu$ L of ABTS solution for 3 min in the dark. Absorbance was measured at 734 nm using a UV/Vis spectrophotometer. A blank was prepared without the extract. Ascorbic acid was used as a standard. The scavenging activity was derived following Eq. (1):

$$\text{Inhibition of ABTS activity (\%)} \\ = [1 - (\text{A}_{734 \text{ nm sample}} / \text{A}_{734 \text{ nm, blank}})] \times 100 \quad (1)$$

1,1-Diphenyl-2-picryl-hydrazil (DPPH) radical scavenging was evaluated as described by Brand-Williams et al. [24]. Each sample extract was mixed with 25% methanol and 0.1 mM DPPH solution. After 30 min in the dark, the absorbance of the mixture was measured at 517 nm. The inhibition percentage of DPPH absorbance was calculated using Eq. (2):

$$\text{Inhibition of DPPH activity (\%)} \\ = [1 - (\text{A}_{517 \text{ nm sample}} / \text{A}_{517 \text{ nm blank}})] \times 100 \quad (2)$$

Inhibition of nitric oxide (NO) was determined according to the method of Marcocci et al. [25]. The extract was heated to 37 °C for 1 h. The incubated solution was mixed with Griess reagent (1% sulfanilamide, 2% H<sub>3</sub>PO<sub>4</sub>, and 0.1% naphthyl ethylenediamine dihydrochloride). The mixture was then incubated for 5 min in the dark and absorbance was measured at 540 nm. NO inhibition was calculated based on Eq. (3):

$$\text{Inhibition of NO activity (\%)} \\ = [1 - (\text{A}_{540 \text{ nm sample}} / \text{A}_{540 \text{ nm blank}})] \times 100 \quad (3)$$

The Fe<sup>2+</sup> chelating activity of the extract was determined as described previously [26]. The reaction mixture contained 1 mL of each extract solution with 3.7 mL of methanol and 0.1 mL of 2 mM ferrous chloride. Subsequently, 0.2 mL of 5 mM ferrozine was added to the mixture and reacted for 10 min at room temperature. The Fe<sup>2+</sup> chelating activity of the solution was measured spectrophotometrically at 562 nm and expressed according to Eq. (4):

$$\text{Inhibition Fe}^{2+} \text{ chelating activity (\%)} \\ = [1 - (\text{A}_{562 \text{ nm sample}} / \text{A}_{562 \text{ nm blank}})] \times 100 \quad (4)$$

### Statistical analyses

All data were collected in triplicate and the means were used for statistical analysis. Analysis of variance and Duncan's multiple range post-hoc test were performed using Statistical Analysis System software (SAS 9.0, SAS Institute, Cary, NC, USA) with a confidence interval of 95% ( $p < 0.05$ ) and 99% ( $p < 0.01$ ).

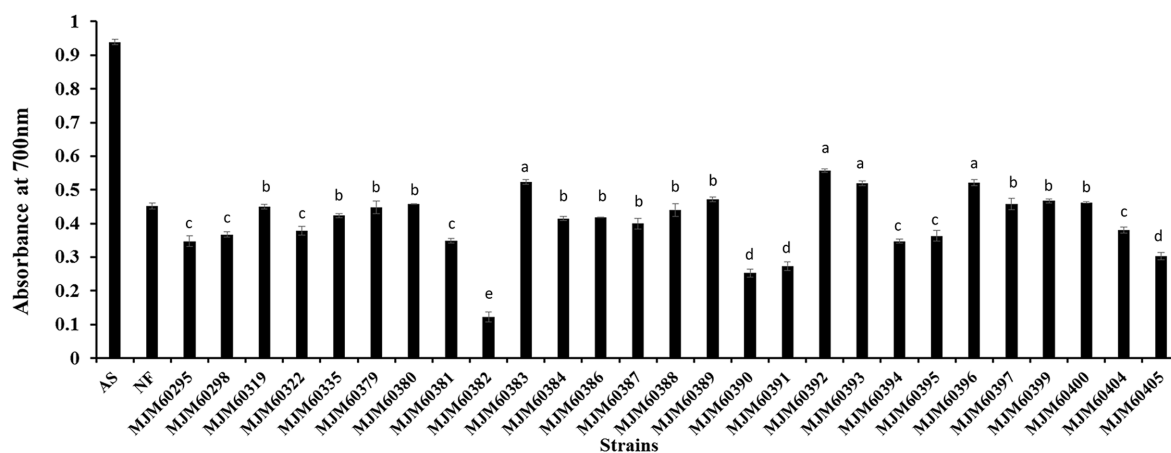
## Results and Discussion

### Isolation of LAB and reducing power of LAB

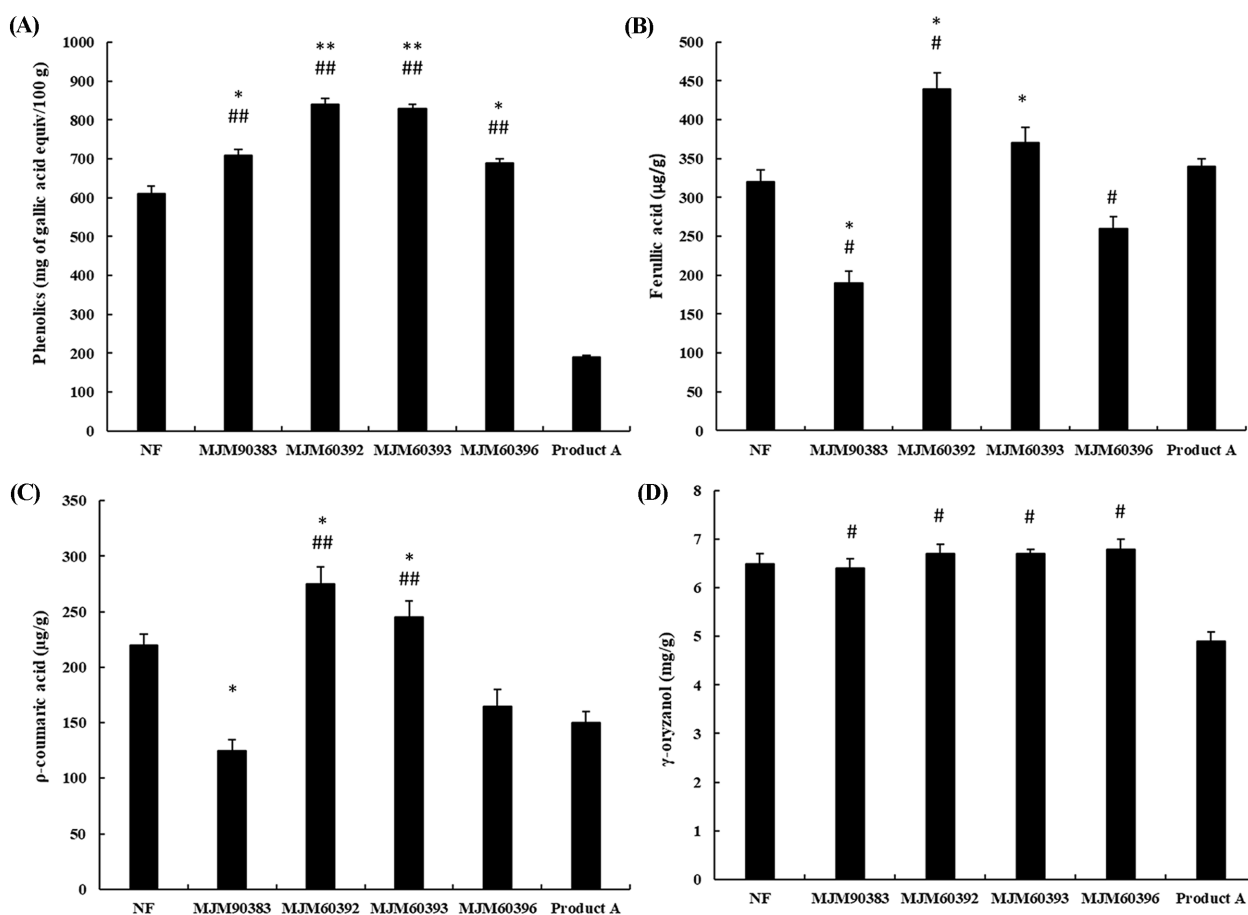
To select the starter rice bran culture candidates, 150 LAB strains isolated from several fermented sources were evaluated. Of these, 27 strains passed safety tests such as biogenic amine production, hemolytic activity, and mucin degradation analyses. These strains were further tested for their reducing power activities. Figure 1 shows the reducing power activities of these strains; four strains, *L. plantarum* MJM60383, *L. lactis* subsp. *lactis* MJM60392, *L. fermentum* MJM60393, and *L. paracasei* MJM60396, showed high activities and were selected as potential starter strains for rice bran fermentation. Previous studies have shown that lactobacilli have strong antioxidant activities; however, the reducing capacity of LAB varies considerably among different strains depending on their metabolites [27,28]. Some LAB strains such as *L. plantarum* can produce L-3-(4-hydroxyphenyl) lactic acid and L-indole-3-lactic acid, which exhibit radical scavenging activity [29].

### Total phenolic content

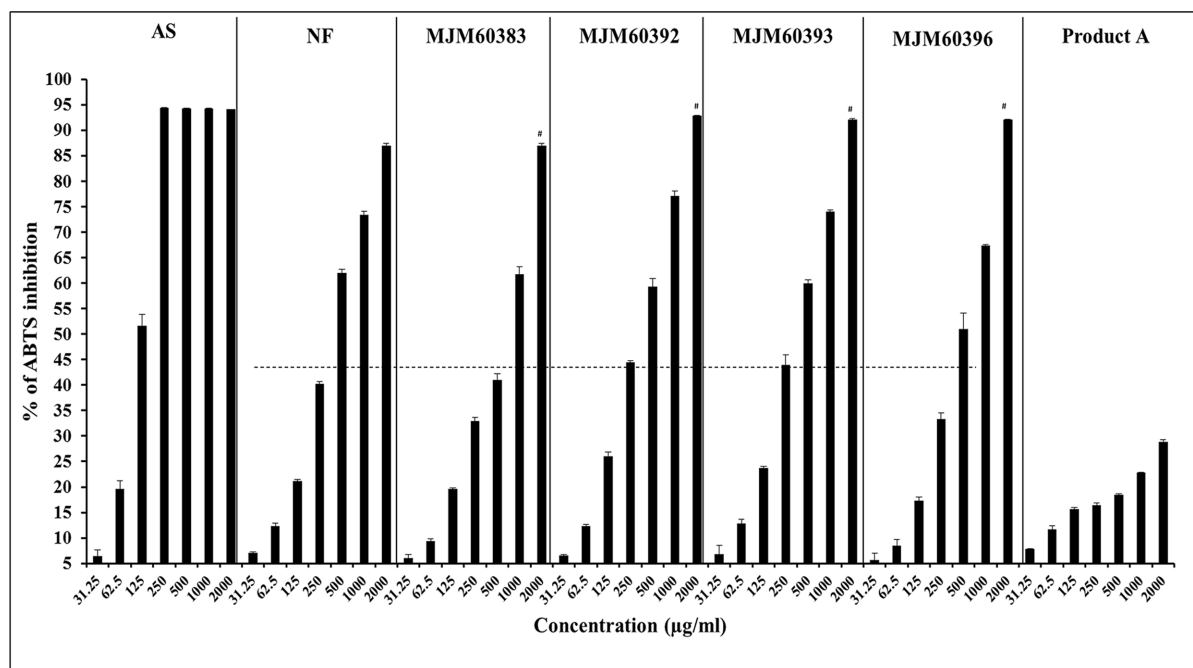
The total phenolic content was determined based on the Folin-Ciocalteu reagent method and the results were expressed as gallic acid equivalents (GAEs) (Fig. 2A). The non-fermented rice bran showed a phenolic compound content of 609.44 mg GAE/g, which was lower than that in the rice bran fermented with all four strains. Rice bran fermented with *Lactococcus lactis* subsp. *lactis* MJM60392 contained the highest phenolic content of 844.13 mg GAE/g compared to 186.25 mg GAE/g in product A. This is similar to the value reported for rice bran fermented with *Rhizopus oryzae* [30]. The increased phenolic content can be explained by



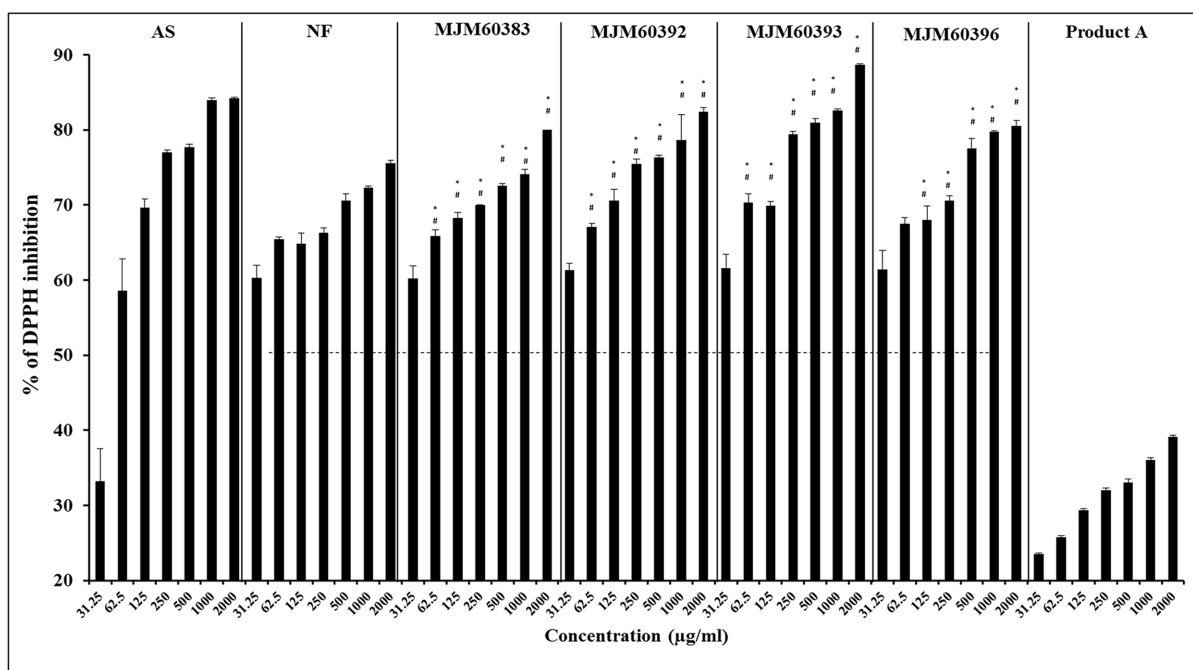
**Fig. 1** Screening strains for reducing power of lactic acid bacteria. The data are presented as the mean  $\pm$  SD ( $n=3$ ). Values with different superscripts are significantly different at  $p < 0.05$ . AS, ascorbic acid; NF, non-fermented rice bran



**Fig. 2** Bioactive compounds content by phenolic extracts of fermented rice bran with different lactic acid bacteria. (A) Total phenolic contents, (B) bound ferulic acids, (C)  $p$ -coumaric acid, and (D)  $\gamma$ -oryzanol acid. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the NF; # $p < 0.05$ , ## $p < 0.01$  vs. product A ( $n=3$ ). NF, non-fermented rice bran



**Fig. 3** ABTS free radical scavenging activity by phenolic extracts of fermented rice bran with different lactic acid bacteria. \* $p < 0.05$  vs. the NF; # $p < 0.05$  vs. product A ( $n=3$ ). AS, ascorbic acid; NF, non-fermented rice bran



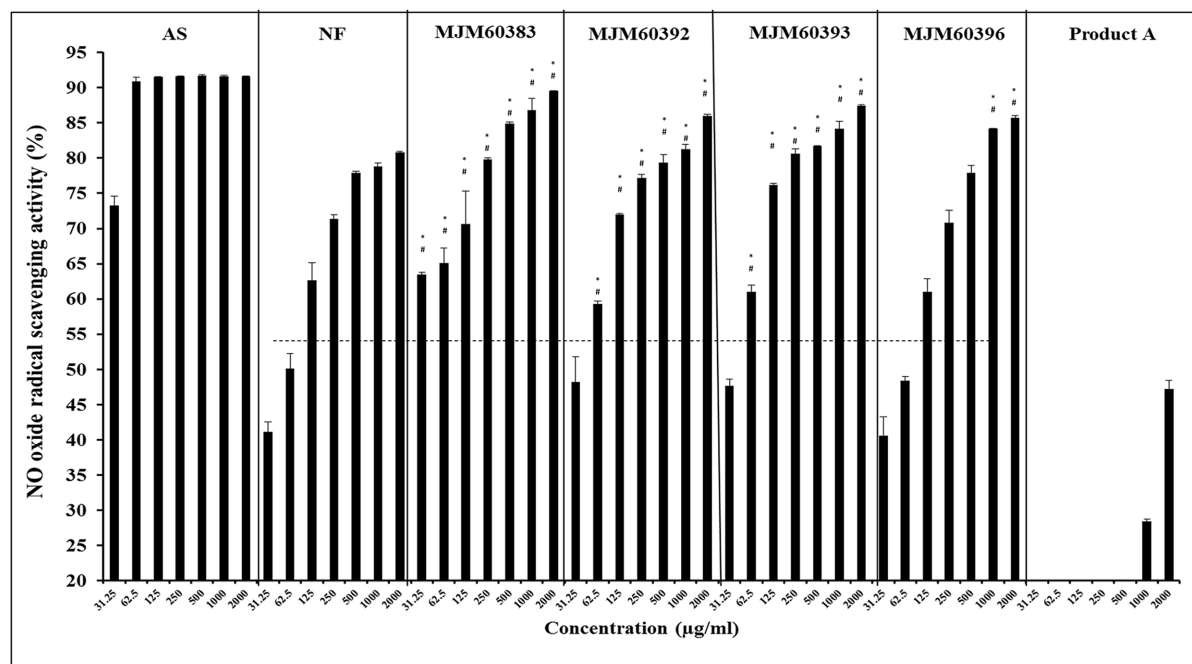
**Fig. 4** DPPH free radical scavenging activity by phenolic extracts of fermented rice bran with different lactic acid bacteria. \* $p < 0.05$  vs. the NF; # $p < 0.05$  vs. product A ( $n=3$ ). AS, ascorbic acid; NF, non-fermented rice bran

the higher levels of  $\beta$ -glucosidase enzymes and esterases produced by LAB during fermentation [15,9,18].

#### Ferulic acids, $p$ -coumaric acid, and $\gamma$ -oryzanol

Ferulic acids,  $p$ -coumaric acid, and  $\gamma$ -oryzanol positively affect

antioxidant activity. The content of these compounds was determined by HPLC analysis and the results are shown in Fig. 2B-D. The content of ferulic acids,  $p$ -coumaric acid, and  $\gamma$ -oryzanol was higher in the rice bran fermented with *L. lactis* subsp. *lactis* MJM60392 and *L. fermentum* MJM60393 than in



**Fig. 5** Nitric oxide (NO) scavenging activity by phenolic extracts of fermented rice bran with different lactic acid bacteria. \* $p < 0.05$  vs. the NF; # $p < 0.05$  vs. product A ( $n=3$ ). AS, ascorbic acid; NF, non-fermented rice bran

other samples. Ferulic acid is an extremely abundant hydroxycinnamic acid in the plant cell wall and shows the highest antioxidant activity [31]. Some microorganisms such as *Aspergillus niger* and *Lactobacillus acidophilus* can produce feruloyl esterase to degrade the esterified ferulate for bioconversion to [15]. These results suggest that rice bran fermented with *L. lactis* subsp. *lactis* and *L. fermentum* contains ferulic acid resulting from feruloyl esterase production. The other predominant phenolic acid  $p$ -coumaric acid was also increased during the fermentation process.

#### Antioxidant activities of LAB-fermented rice bran

The ABTS free radical scavenging activities of rice bran fermented with different LAB strains are shown in Fig. 3. The ABTS scavenging rate increased with increasing extract concentrations. However, there were no significant differences in the ABTS scavenging activities among non-fermented and fermented rice bran. Sirilun et al (2017) reported that ABTS scavenging activity did not differ significantly in LAB-mediated soybean fermentation but increased the phenolic content including isoflavone.

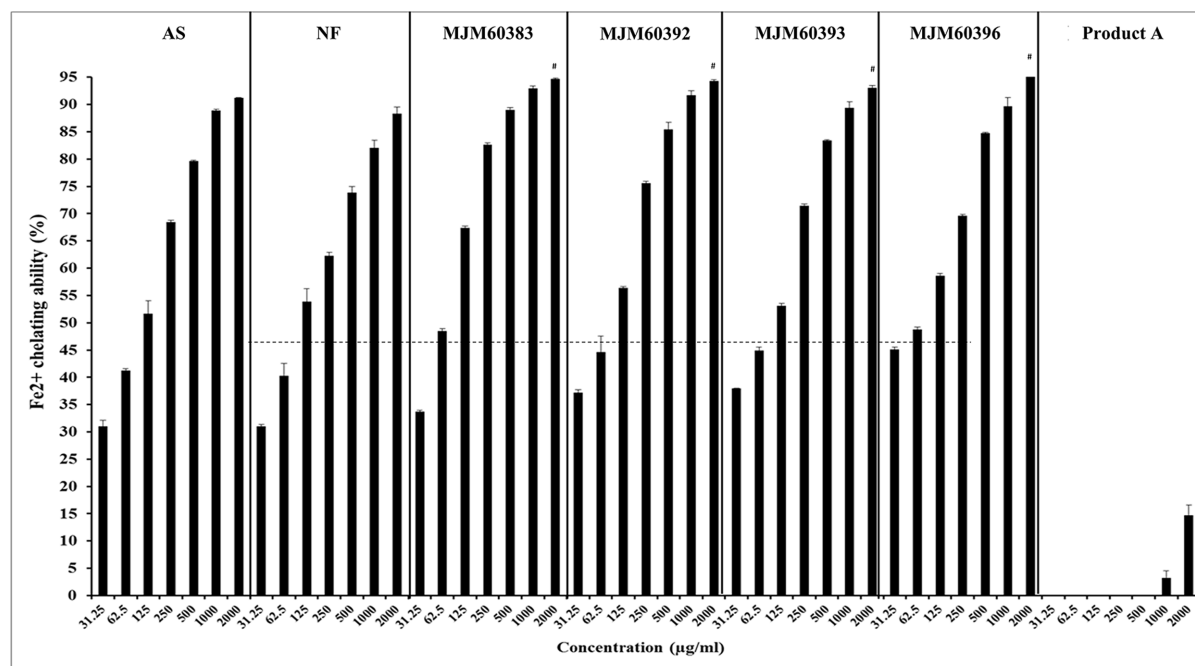
DPPH radical scavenging assays are based on the transfer of electrons from a donor molecule to a corresponding radical. This is the simplest method for measuring the ability of antioxidants to intercept free radicals. All fermented rice bran extracts showed higher activity than the non-fermented rice bran extracts (Fig. 4). Rice bran fermented with *Lactobacillus fermentum* MJM60393 showed the highest (88.64%) DPPH free radical scavenging activity, which was more than 1.2-fold that of the non-fermented rice bran (75.55%). The DPPH radical scavenging activities

determined in our study are similar to those of most fermented rice brans [32]. In fact, the DPPH radical scavenging activity showed a strong correlation with phenolic compounds [33]. This finding agrees with the results of increased content of ferulic acids and  $p$ -coumaric, which also showed that the LAB culture fermented with rice bran had strong antioxidant activity.

The free radical scavenging activities of all extracts were determined by measuring NO (nitric oxide) scavenging activity and using ascorbic acid as a standard. As shown in Fig. 5, the NO activity of all fermented rice brans was higher than that of non-fermented rice bran. *Lactobacillus plantarum* MJM60383 showed the highest activity at 2000  $\mu\text{g/mL}$  (89.54%). This sample showed a value 1.1-fold higher than that of non-fermented rice bran (80.82%). The NO scavenging activities of *L. lactis* subsp. *lactis* MJM60392, *L. fermentum* MJM60393, and *L. paracasei* MJM60396 were 85.98, 87.43, and 85.70%, respectively.

Chelation of  $\text{Fe}^{2+}$  by extracts was estimated as described by Dinis et al. (1994) and the results are shown in Fig. 6. There were no significant differences with the results among the four strains. The results suggest that the antioxidant activity of rice bran and fermented rice brans is not related to the iron-binding capacity.

In summary, fermenting rice bran with LAB is an effective method for increasing antioxidant activity. Among these strains, *L. lactis* subsp. *lactis* MJM60392 produced large amounts of phenolic compounds compared to the other strains tested, leading to high antioxidant activities. Thus, LAB can be applied in functional foods to improve human health.



**Fig. 6** Fe<sup>2+</sup> chelating ability by phenolic extracts of fermented rice bran with different lactic acid bacteria. <sup>#</sup>*p* < 0.05 vs. product A (n=3). AS, ascorbic acid; NF, non-fermented rice bran

**Acknowledgment** This study was carried out with the support of the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A3 B03027816).

**Conflict of interest statement** We declare that we have no conflicts of interest.

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