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Physicochemical Properties and Bioactivities of Rice Beans Fermented by *Bacillus amyloliquefaciens*

Shan Wu [a], Shuai Lu [b], Jun Liu [b], Shaoqing Yang [b], Qiaojuan Yan [a,#,*], Zhengqiang Jiang [b,#,*]

[a] Key Laboratory of Food Bioengineering (China National Light Industry), College of Engineering, China Agricultural University, Beijing 100083, China
[b] Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

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ABSTRACT

The purpose of this study was to investigate the physicochemical properties and bioactivities of rice beans (*Vigna umbellata*) fermented by *Bacillus amyloliquefaciens*. The fermentation conditions were optimized on the basis of the fibrinolytic activity. Under the optimal fermentation conditions, the fibrinolytic activity reached a maximum of 78.0 FU·g^{-1} (4890 IU·g^{-1}, fibrin plate method, FU: fibrin degradation unit). The contents of peptides (which increased from 2.1 to 10.9 g per 100 g), total phenolics (from 116.7 to 388.5 mg gallic acid per 100 g), total flavonoids (from 235.5 to 354.3 mg rutin per 100 g), and anthocyanin (from 20.1 to 47.1 mg per 100 g), as well as the superoxide dismutase activity (from 55.3 to 263.6 U·g^{-1}) in rice beans were significantly increased after fermentation. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) free radical scavenging activities and ferric reducing antioxidant power (FRAP) of fermented rice beans were 1.9–4.8 times higher than those of unfermented rice beans. Moreover, fermentation induced an increase in the dipeptidyl peptidase IV (DPP-IV) inhibition, α-glucosidase inhibition, and anticoagulant activities of rice beans. Rice beans fermented by *Bacillus amyloliquefaciens* may serve as a functional food with potential benefits for the prevention of thrombotic diseases.

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1. Introduction

Cardiovascular disease (CVD) is an important factor affecting human health, and accounts for approximately one-third of deaths worldwide. Strokes and coronary heart disease are primarily caused by obstructions in the blood vessels that preclude blood from flowing into the brain or heart. Therefore, thrombolytic therapy is an effective way to prevent CVD [1]. During the past decades, many researchers have focused on safe and cheap antithrombotic foods. Studies have reported that fermentation enhances the fibrinolytic activity of soy foods, including Chinese dochi, Japanese natto, and Korean soybean pastes [2]. Fermented soy foods have significant modifications in flavor and texture due to the action of microorganisms [3]. Bacteria, especially *Bacillus* sp., have been recognized as the most important genus producing fibrinolytic enzymes. Some fibrinolytic enzymes have been discovered from food-grade bacteria such as *Bacillus subtilis* (*B. subtilis*) [4], *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) [5–7], *Bacillus vallismortis* Ace02 [8], and *B. megaterium* KSK-07 [9]. Soybeans fermented by *B. subtilis* have antithrombotic effects both *in vitro* and *in vivo* [4,10]. *B. amyloliquefaciens* is close to *B. subtilis*, and can be screened from Chinese dochi and Korean soy sauce [5,10]. Subtilisins, a fibrinolytic enzyme, was found to be produced by *B. amyloliquefaciens* [7]. Solid-state fermentation is considered to be a good process for the promotion of nutrients and active substances, as well as antioxidant activity, in many legumes and cereals [11]. However, there are very few reports on the thrombolytic and anticoagulant activities of solid-state fermented foods by *B. amyloliquefaciens* [6].

The rice bean is a legume with strong viability that possesses drought resistance, a short growth period, and simple cultivation techniques. This crop originates from tropical Asia and has been cultivated in India, Korea, Japan, and South China. In particular, the levels of nutrients and active substances are higher in rice beans than in many other beans belonging to the same genus [12]. The rice bean is rich in phenolic compounds and shows bioactivities that include antioxidant activity and antidiabetic potential [13]. Furthermore, due to its low recognition and high nutritional

* Corresponding authors.
E-mail addresses: yanqj@cau.edu.cn (Q. Yan), zhqjiang@cau.edu.cn (Z. Jiang).
# These authors contributed equally to this work.

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quality, the rice bean has great development potential in the food market [14]. Solid-state fermentation has the advantages of high productivity, low energy consumption, easy control of the fermentation process, and low sterility requirements [15]. In the present study, rice beans were subjected to solid-state fermentation using *B. amyloliquefaciens*. The effects of fermentation on the physicochemical properties and bioactivities of the antioxidant, antidiabetic, and antithrombotic activities of the rice beans were evaluated.

2. Materials and methods

2.1. Materials, chemicals, and microorganisms

Rice beans were purchased from a supermarket in Beijing, China, and were stored at 4 °C until used. Folin–Ciocalteu phenol reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), ferric tripyridyltriazine (TPTZ), urokinase from human kidney cells (10 KU), thrombin from bovine plasma, fibrinogen, and dipeptidyl peptidase IV (DPP-IV) were bought from Sigma-Aldrich (Canada). Heparin sodium, rutin, and acarbose were supplied by BioDee Biotechnology Co., Ltd. (China).

B. amyloliquefaciens CAUNDJ118 was screened from mushroom sauce and assigned a preservation number (CGMCC NO. 16050) in the China General Microbiological Culture Collection Center (CGMCC).

2.2. Fermentation and extraction of rice beans

Fifty grams of rice beans were soaked in deionized water for 18 h. The swollen beans were then placed on a plate and cooked at 121 °C. The cooked beans were cooled, inoculated with various concentrations of bacteria, and incubated at 40 °C to optimize the fermentation conditions on the basis of fibrinolytic activity. Un-inoculated rice beans were used as the blank control (unfermented rice beans). Both the fermented and unfermented samples were freeze-dried and then extracted separately with deionized water (1/5 (v/v)), 80% (v/v), ethanol, and 0.5% HCl in methanol, respectively. The extracts were collected and stored at −20 °C until subsequent analyses.

2.3. Measurement of fibrinolytic activity

Fibrinolytic activity was evaluated by two methods. One was the fibrin degradation method, which was applied based on the protocols previously described by Lee et al. [16]. In brief, 1.4 mL of 50 mmol·L^{-1} KCl-H_3BO_3 buffer (pH 8.0) and 0.4 mL of 0.72% fibrinogen were incubated at 37 °C for 5 min. Next, 0.1 mL of thrombin (20 U·mL^{-1}) was added to react at 37 °C for 10 min. Subsequently, 0.1 mL of sample water extract was added and incubated at 37 °C for 60 min. The reaction was stopped with 2 mL of 0.2 mol·L^{-1} trichloroacetic acid (TCA), and the absorption value was measured at 275 nm after centrifugation for 10 min (10 000 r·min^{-1}). One unit (fibrin degradation unit, FU) of enzyme activity is defined as the amount of enzyme producing a 0.01 increment of absorbance at 275 nm per minute. The fibrinolytic activity was expressed as FU·g^{-1} fresh weight.

The other method was the fibrin plate method, which was employed to measure fibrinolytic activity as previously described by Feng et al. [17], with slight modifications. The fibrin plate was prepared by decanting a mixture that incorporated 5 mL of 0.4% fibrinogen in boric acid-borate buffer with 5 mL of 0.8% agarose and 1 mL of thrombin (200 U·mL^{-1}). To quantify the fibrinolytic activity of the samples, urokinase solution with different activity units was used as the standard curve. The results were expressed as IU urokinase per gram fresh weight.

2.4. Physical properties of rice beans

The hardness of unfermented and fermented rice beans by *B. amyloliquefaciens* was analyzed according to the method previously described by Shih et al. [18], with slight modifications. Unfermented and fermented rice beans were equilibrated to 25 °C before measurement, and the hardness was determined by means of a texture analyzer (TMS-PRO; FTC, USA). A 25 000 g load cell was applied and the moving speed was set at 1 mm·s^{-1}. In addition, a plunger probe (ϕ38.1 mm) was applied to press the beans to 60% of the original height. Each sample had nine replicates. The maximum force was considered as the hardness (g). The dynamic viscosity of the rice beans was determined with a viscometer (DV-1 Viscometer; Yueping, China). Ten grams of rice beans were shaken with 100 mL deionized water for 30 min and the slimy substances were extracted. Next, 20 mL of the extracts was put into the beaker and the viscosity was measured at 30 °C. The microstructure was examined with a scanning electron microscope (SEM, S-3400N; HITACHI, Japan). The sample was covered with gold and observed at a voltage of 5 kV with 1000× magnification.

2.5. Chemical properties of rice beans

Reducing sugar content was determined by the 3,5-dinitrosalicylic acid (DNS) method [19] and expressed in milligrams of glucose per gram of dry rice beans. Peptide content was measured using the *o*-phthaldialdehyde method with Gly-Leu as the standard [20]. Total phenolic content (TPC) and total flavonoid content in the ethanol extracts were analyzed using the Folin–Ciocalteu method and the colorimetric method, respectively [13]. Anthocyanin content in the 0.5% HCl methanol extracts was determined using the pH differential method as described by Chiou et al. [21]. Superoxide dismutase (SOD) activity in the water extracts was measured using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China). Powdered samples were treated by acidic hydrolysis. Amino acid composition analysis was performed using the Hitachi L-8900 amino acid analyzer with ion-exchange chromatography and the post-column ninhydrin derivation method [22]. Amino acid contents were quantified against 0.2 mmol·L^{-1} working standard solutions for 17 kinds of amino acids. The results were recorded in mg·g^{-1} dry weight.

2.6. Measurement of antioxidant, α-glucosidase inhibition, DPP-IV inhibition, and anticoagulant activities

The antioxidant activity of both the water extracts and the ethanol extracts of the samples was analyzed. DPPH and ABTS free radical scavenging activities and ferric reducing antioxidant power (FRAP) were determined as reported by Dudonné et al. [23]. All the results were expressed as μmol Trolox per gram dry weight.

The α-glucosidase inhibition activity of the ethanol extracts was determined according to the method previously described by Shukla et al. [24], with slight modifications. In brief, the reaction solution, including phosphate buffer (0.1 mol·L^{-1}, pH 6.8, 50 μL), sample (30 μL), and α-glucosidase (1.5 U·mL^{-1}, 30 μL), was incubated at 37 °C for 10 min. Subsequently, *p*-nitrophenyl-α-*D*-glucopyranoside (0.5 mmol·L^{-1}, 40 μL) was added to the solution, and the resulting mixture was maintained at 37 °C for 40 min. Na_2CO_3 reagent (0.2 mol·L^{-1}, 80 μL) was used to terminate the enzymatic reaction. The solution without extracts was used as the control and the solution without α-glucosidase was used as

the blank control. Absorbance at 405 nm was measured and the inhibitory percentage was calculated as follows:

$$\alpha\text{-glucosidase inhibition activity} = (A_{control} - A_{sample})/(A_{control} - A_{controlblank}) \times 100\% \quad (1)$$

where $A_{control}$ is the absorbance of the solution without a sample, A_{sample} is the absorbance of the samples, and $A_{controlblank}$ is the absorbance of the blank solution without α-glucosidase.

The DPP-IV inhibitory activity of the water extracts was measured by the method reported by Wang et al. [25]. In brief, the extracts were diluted with 0.1 mol·L^{-1} Tris-HCl buffer (pH 8.0). The sample solution (25 μL) was combined with 25 μL 1.6 mmol·L^{-1} Gly-Pro-*p*-nitroanilide. The reaction solution was incubated at 37 °C for 10 min. Then, 50 μL DPP-IV (8 U·L^{-1}) was added. The reaction solution was placed at 37 °C for 60 min and 100 μL sodium acetate buffer (1 mol·L^{-1}, pH 4.0) was used to stop the reaction. Absorbance at 405 nm was measured and the DPP-IV inhibitory activity was determined as follows:

$$\text{DPP-IV inhibition activity} = [(A_B - A_{BC}) - (A_S - A_{SC})]/(A_B - A_{BC}) \times 100\% \quad (2)$$

where A_B is the absorbance of the mixture without extracts (blank group); A_{BC} and A_{SC} is the absorbance of the blank mixture and the extracts mixture without DPP-IV, respectively; and A_S is the absorbance of the reaction mixture (the sample group or the positive group).

Measurement of anticoagulant activity was performed in 96-well microplates [26]. The inhibition abilities were calculated according to the following equation:

$$\text{Anticoagulant activity} = [1 - (A_S - A_{SB})/(A_C - A_{CB})] \times 100\% \quad (3)$$

where A_C is the absorbance of the mixture without sample (buffer replaces the sample); and A_{CB} and A_{SB} is the absorbance of the control mixture and the sample mixture without thrombin (buffer replaces the thrombin), respectively.

2.7. Statistical analysis

All experiments were performed at least in triplicate. The results were reported as the mean ± standard deviations. Statistical differences were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests with SPSS 20.0 software (SPSS Chicago, IL, USA). A *p* value below 0.05 was considered to be significant.

3. Results and discussion

3.1. Optimization of fermentation conditions

Rice beans were steamed for 10–50 min and inoculated with *B. amyloliquefaciens* at a viable count of 1 × 10^5 CFU (CFU: colony forming unit) per 100 g. After solid-state fermentation for 24 h, the physicochemical properties and fibrinolytic activity of the fermented rice beans were analyzed. As shown in Fig. 1(a), the hardness decreased (from 2798 to 1058 g) and the fibrinolytic activity and dynamic viscosity increased with an increase in steaming time. The maximum fibrinolytic activity (73.7 FU·g^{-1}) and dynamic viscosity (68.0 mPa·s) of the fermented rice beans were obtained after 40 min of steaming. Furthermore, the effects of the inoculation amount and fermentation time on the fibrinolytic activity, hardness, and dynamic viscosity for 40 min of steaming time were investigated. As shown in Figs. 1(b) and (c), when the viable count was 10^7 CFU per 100 g and the fermentation time was 24 h, the

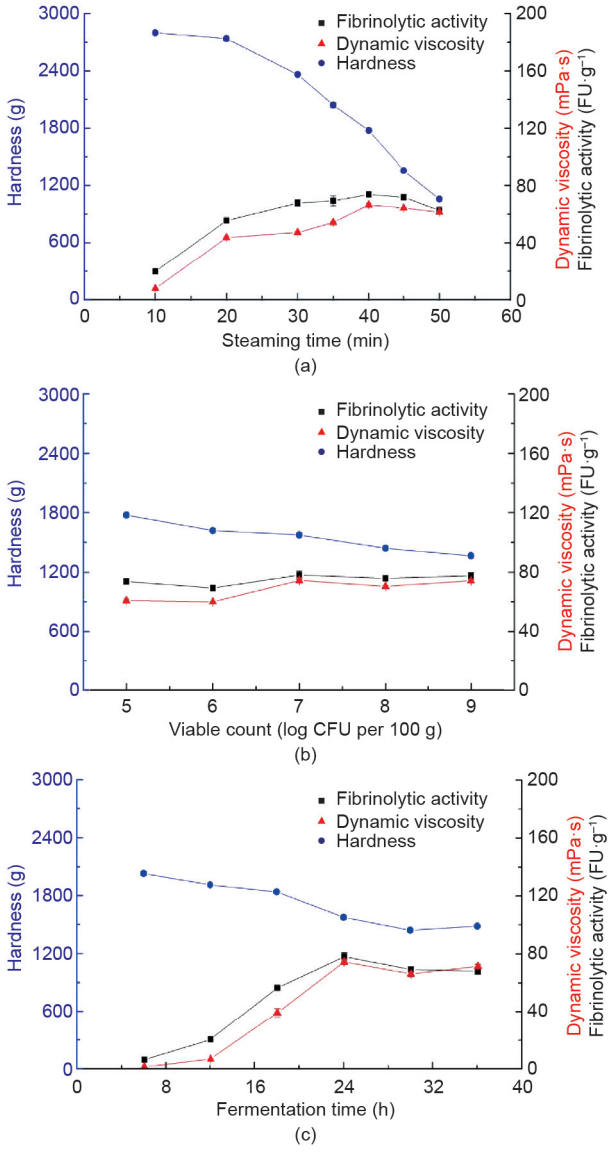

Fig. 1. (a) Effect of steaming time, (b) viable count, and (c) fermentation time on the fibrinolytic activity, dynamic viscosity, and hardness of rice beans fermented by *Bacillus amyloliquefaciens*.

maximum fibrinolytic activity and dynamic viscosity were 78.0 FU·g^{-1} (4890 IU·g^{-1}, fibrin plate method) and 76.0 mPa·s, respectively. The sensory evaluation, stringiness, and flavor of the fermented rice beans were very similar to those of commercially available natto, which is a Japanese traditional fermented soybean product [10]. Moreover, the "ammonia" smell of the fermented rice beans was much less than that of natto.

Under the optimized fermentation conditions, the maximum fibrinolytic activity of the fermented rice beans was found to be 78.0 FU·g^{-1} (4890 IU·g^{-1}, fibrin plate method) in the present study. Based on our knowledge, there is no standard method for the determination of fibrinolytic activity. Both the fibrin degradation method and the fibrin plate method are commonly used for fibrinolytic activity analysis. In comparison with the fibrin degradation method, the fibrin plate method is easier to operate but has lower sensitivity. In comparison with reported data, the fibrinolytic activity of the rice beans fermented by *B. amyloliquefaciens* was measured by both the fibrin degradation method and the fibrin plate method. In general, commercially available natto exhibits a fibrinolytic activity of 20–40 FU·g^{-1} [27]. In addition, many other

grains have been reported to have fibrinolytic activity. In the solid-state fermentation of chickpeas, the fibrinolytic activity has been reported to reach 39.3 FU·g^{-1} under the optimized conditions [6]. Some studies have reported that other fermented beans have fibrinolytic activities, such as fermented red beans with *Bacillus* sp. and *Lactobacillus delbrueckii* subsp. (28.2 FU·g^{-1}) [2], and pigeon peas fermented by *B. subtilis* (1895.1 IU·g^{-1}, 53.0 FU·g^{-1}) [16,17]. Thus, the fibrinolytic activity of the rice beans fermented by *B. amyloliquefaciens* is much higher than those of commercial natto and other fermented grains.

3.2. Physicochemical properties of rice beans fermented by B. amyloliquefaciens

Compared with unfermented rice beans, the hardness (2194.6 and 1575.0 g) was decreased and the moisture content (60.5% and 64.6%), dynamic viscosity (0.5 and 65.8 mPa·s), reducing sugar content (1.9 and 13.9 g glucose per 100 g dry weight (dw)), and peptide content (2.1 and 10.9 g per 100 g dw) were increased in rice beans fermented under the optimized fermentation conditions (Table 1). The physicochemical properties of the rice beans were significantly changed after fermentation by *B. amyloliquefaciens*. To be specific, the dynamic viscosity of the fermented rice beans was much higher (131.6 times) than that of the unfermented rice beans. Hu et al. [28] and Shih et al. [18] found that the viscosity of black soybeans increased during the fermentation process, while the hardness decreased. Gamma-polyglutamic acid was found to be one of the major components in the sticky silk of fermented soybeans [29]. It has been suggested that the viscosity of beans fermented by *Bacillus* strains is an important indicator for estimating fibrinolytic activity. Our results are in agreement with the findings of Shih et al. [18], which indicate that dynamic viscosity is positively correlated with fibrinolytic activity (correlation coefficient r = 0.986; p < 0.05, Fig. 1). Overall, viscous substances with fibrinolytic activity were produced during the fermentation of rice beans using *B. amyloliquefaciens*.

The total phenolic (116.7 and 388.5 mg per 100 g), total flavonoid (235.5 and 354.2 mg per 100 g), and anthocyanin contents (20.1 and 47.1 mg per 100 g) in the fermented rice beans increased by 1.5–3.3-fold, as compared with the unfermented rice beans (Table 1). Phenolic acids and flavonoids (20–1000 mg per day) are taken by some people depending on their dietary sources and habits [30]. Phenolic compounds have been recognized as high-profile substances in recent years due to their potential bioactivities. Many reports have shown that fermentation increases the phenolic content of beans due to the release of free phenol aglycones. TPC has been shown to increase in black soybeans (1.5 times), chickpeas (3.2 times), soybeans (1.7 times), and pigeon peas (1.4 times) during fermentation [6,16,31,32]. In black soybeans fermented by *B. subtilis*, β-glucosidase was produced and released the phenolic hydroxyl group [6]. The TPC of rice beans fermented by *B. amyloliquefaciens* increased by 3.3 times (Table 1) [6,16,31,32]. Phenolic compounds are commonly present in raw-food material in binding forms, and the bioactivities of bound phenols are usually lower than those of free phenols [31,33]. In this study, *B. amyloliquefaciens* was capable of producing β-glucosidase (from 0.01 to 2.19 U·g^{-1}, data not shown) in rice beans. Thus, it is possible that the increase of TPC in fermented rice beans is caused by a release of cell-wall-bound phenolics [23,31].

As shown in Table 1, the SOD activity of the unfermented and fermented rice beans ranged from 55.3 to 263.6 U·g^{-1} dw. The fermented rice beans had significantly higher SOD activity than the unfermented rice beans. As presented in Table 1, the SOD activity of the rice beans increased by 4.8 times after fermentation by *B. amyloliquefaciens*. SOD has special physiological activity and is the primary substance for scavenging free radicals in organisms [34]. Improvement in SOD activity has also been detected in natto and fermented black soybeans [35,36].

The amino acid composition of rice beans fermented by *B. amyloliquefaciens* was compared with that of unfermented rice beans (Table 2). Both types of rice beans were rich in glutamic acid (34.8 and 42.4 mg·g^{-1}) and aspartic acid (24.8 and 24.2 mg·g^{-1}), as well as the essential amino acids leucine (17.7 and 17.9 mg·g^{-1}) and lysine (16.4 and 16.4 mg·g^{-1}). After fermentation, the content of glutamic acid, alanine, cysteine, methionine, and phenylalanine in the fermented rice beans was increased by 21.8%, 7.8%, 35.0%, 38.1%, and 3.2%, respectively. However, *B. amyloliquefacien* fermentation induced the decrease of arginine content in rice beans (7.8%).

SEM images of the rice beans before and after fermentation are shown in Fig. 2(a). Significant differences were observed in the surface microstructures of the unfermented rice beans and fermented rice beans in the SEM images. The surface structure of the unfermented rice beans was rough, but intact and tight (Fig. 2(a)). However, the external skeleton structure of the rice beans was destroyed and a large area on each bean was exfoliated after fermentation, forming a great deal of debris, and a rough and loose shape with inhomogeneous pores (Fig. 2(b)).

Table 1
Physicochemical properties of unfermented and fermented rice beans.

Physicochemical properties	Unfermented rice beans	Fermented rice beans
Moisture content (%)	60.5^{b} ± 0.7	64.6^{a} ± 0.3
Hardness (g)	2194.6^{a} ± 31.3	1575.0^{b} ± 46.1
Dynamic viscosity (mPa·s)	0.5^{b} ± 0.1	65.8^{a} ± 3.1
Reducing sugar (g glucose per 100 g dw)	1.9^{b}	13.9^{a} ± 0.1
Peptide content (g per 100 g dw)	2.1^{b} ± 0.2	10.9^{a} ± 0.6
SOD activity (U·g^{-1})	55.3^{b} ± 0.8	263.6^{a} ± 1.0
TPC (mg gallic acid per 100 g dw)	116.7^{b} ± 1.4	388.5^{a} ± 4.9
TFC (mg rutin per 100 g dw)	235.5^{b} ± 3.5	354.3^{a} ± 4.5
Anthocyanins content (mg per 100 g dw)	20.1^{b} ± 1.9	47.1^{a} ± 1.6

Different letters (a, b) in the same row are significant different at p < 0.05.
TFC: total flavonoid content.

Table 2
Amino acid compositions of unfermented and fermented rice beans.

Amino acid	Unfermented rice beans	Fermented rice beans
Aspartic acid	24.8^{a} ± 0.4	24.2^{a} ± 0.2
Threonine†	7.3^{a} ± 0.3	7.4^{a} ± 0.2
Serine	8.8^{a} ± 0.7	8.6^{a} ± 0.6
Glutamic acid	34.8^{b} ± 0.5	42.4^{a} ± 0.4
Proline	9.3^{a} ± 0.4	9.6^{a} ± 0.1
Glycine	8.7^{a}	8.9^{a} ± 0.2
Alanine	10.3^{b} ± 0.2	11.1^{a}
Cysteine	2.0^{b} ± 0.1	2.7^{a} ± 0.4
Valine†	12.1^{a} ± 0.1	12.7^{a} ± 0.5
Methionine†	2.1^{b}	2.9^{a} ± 0.3
Isoleucine†	10.3^{a} ± 0.1	10.6^{a} ± 0.3
Leucine†	17.7^{a} ± 0.3	17.9^{a}
Tyrosine	5.3^{a}	5.4^{a} ± 0.1
Phenylalanine†	12.6^{b} ± 0.1	13.0^{a}
Lysine†	16.4^{a} ± 0.2	16.4^{a}
Histidine	6.4^{a} ± 0.2	6.2^{a} ± 0.1
Arginine	12.5^{a} ± 0.1	11.6^{b}
Tryptophan†	2.1^{a}	2.1^{a}
Total amino acids	203.2^{b} ± 3.0	213.3^{a} ± 0.5
Sum essential amino acids	67.9^{b} ± 0.6	69.9^{a} ± 0.8
Sum non-essential amino acids	135.0^{b} ± 2.4	143.5^{a} ± 0.3

Different letters (a, b) in the same row are significant different p < 0.05. The unit of the values is mg per gram dw.
†Essential amino acids in adults.

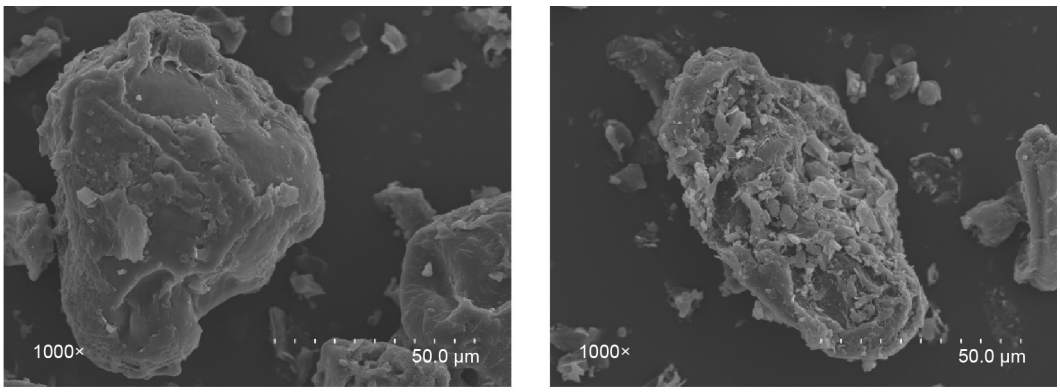

Fig. 2. The scanning electron microscopy of (a) unfermented and (b) fermented rice beans freeze-dried powder at a voltage of 5 kV with 1000× magnification.

3.3. Effect of fermentation on the biological activities of rice beans

3.3.1. Antioxidant activity

Table 3 presents the antioxidant activity of the unfermented and fermented rice beans. The ethanol extracts of the fermented rice beans exhibited the highest DPPH (17.1 μmol Trolox per gram) and ABTS (185.5 μmol Trolox per gram) scavenging activities and FRAP (28.8 μmol Trolox per gram), followed by the water extracts of the fermented rice beans (13.3, 138.3, and 21.0 μmol Trolox per gram, respectively), the ethanol extracts of the unfermented rice beans (6.9, 38.6, and 15.2 μmol Trolox per gram), and the water extracts of the unfermented rice beans (3.9, 29.0, and 10.1 μmol Trolox per gram).

Many studies have focused on radical scavenging effects as an antioxidant model due to the steady repetition and high degree of accuracy of such effects [37]. The DPPH scavenging activity of methanol extracts of chickpeas fermented by *Bacillus* sp. was found to be higher than that of unfermented chickpeas [6]. The DPPH and ABTS scavenging activities, as well as the FRAP, of aqueous extracts of pigeon peas were improved by fermentation with *B. subtilis* [16]. After the solid-state fermentation of rice beans by *B. amyloliquefaciens*, the DPPH and ABTS scavenging activities, and the FRAP of both ethanol and water extracts of rice beans were improved by 2.5, 4.8, and 1.9 times and by 3.4, 4.8, 2.1 times, respectively. Moreover, the ethanol extracts of unfermented or fermented rice beans showed a higher level of antioxidant activity than the water extracts in the present study (Table 3). The phenolic compounds of fermented foods are directly associated with the antioxidant activity [37]. Rice beans contain various phenolic acids, with 14 compounds detected in ethanol extracts and 10 compounds detected in aqueous extracts [19]. Owing to the structure variations of phenolic compounds, the phenolic compositions varied with different extraction solvents. This could be responsible for the higher antioxidant activity of the ethanol extracts of rice beans after fermentation by *B. amyloliquefaciens*.

3.3.2. The α-glucosidase inhibition activity

The α-glucosidase inhibition activity of the ethanol extracts of fermented rice beans at a concentration of 11.2 mg·mL^{-1} (88.9%) was 3.2 times higher than that of unfermented rice beans (27.5%, Fig. 3(a)).

The α-glucosidase inhibitors have the potential to manage type-2 diabetes [24]. Polyphenols are known to be inhibitors of α-amylase and α-glucosidase [37]. It has been reported that rice beans exhibit excellent antidiabetic potential [13]. In this study, *B. amyloliquefaciens* fermentation improved the α-glucosidase inhibition activity of the ethanol extracts of rice beans (from 27.5% to 88.9% at 11.2 mg·mL^{-1}; Fig. 3(a)). The bioactive compounds such as phenols and flavonoids in the ethanol extracts of rice beans increased with fermentation (Table 1). Water extracts of fermented and unfermented rice beans did not show α-glucosidase inhibition activity (data not shown). Shukla et al. [24] evaluated the α-glucosidase inhibition activity of the water extracts of several fermented soybean sauces and detected low inhibition activity (58.93%–62.25% at 50 mg·mL^{-1}). The α-glucosidase inhibition activity of fermented rice beans was lower than that of the positive control (Fig. 3(a)), which could be caused by the exceptionally high level of dietary α-glucosidase inhibitors in fermented rice beans.

3.3.3. Dipeptidyl peptidase IV inhibition activity

The DPP-IV inhibition activity of the water extracts of the rice beans is shown in Fig. 3(b). The fermented rice beans showed significantly higher DPP-IV inhibition activities than the unfermented rice beans at different concentrations (e.g., 92.4%, 65.6%, and 30.2% at 6.4 mg·mL^{-1}, respectively).

DPP-IV has been reported to inhibit insulin secretion, thus, is usually used for the prevention and treatment of type-2 diabetes [25]. Several beans, such as cowpeas (*Vigna unguiculata*), common beans (*Phaseolus vulgaris* L.), and bambara beans (*Vigna subterranea*), have been used for the production of DPP-IV inhibition peptides [38–40]. In this study, the DPP-IV inhibition activity of rice

Table 3
Antioxidant activity of unfermented and fermented rice beans.

Antioxidant activity	Unfermented rice beans		Fermented rice beans	
	Water extracts†	Ethanol extracts‡	Water extracts†	Ethanol extracts‡
DPPH assay	3.9^{d} ± 0.3	6.9^{c} ± 0.3	13.3^{b} ± 0.1	17.1^{a} ± 0.1
ABTS assay	29.0^{d} ± 0.5	38.6^{c} ± 1.2	138.3^{b} ± 1.5	185.5^{a} ± 1.7
FRAP assay	10.1^{d} ± 0.4	15.2^{c} ± 0.3	21.0^{b} ± 0.4	28.8^{a} ± 0.6

Different letters (a, b, c, d) in the same row are significant different at $p < 0.05$. The unit of the values is μmol trolox per gram dw.
†Samples were extracted with deionized water.
‡Samples were extracted with 80% (v/v) ethanol.

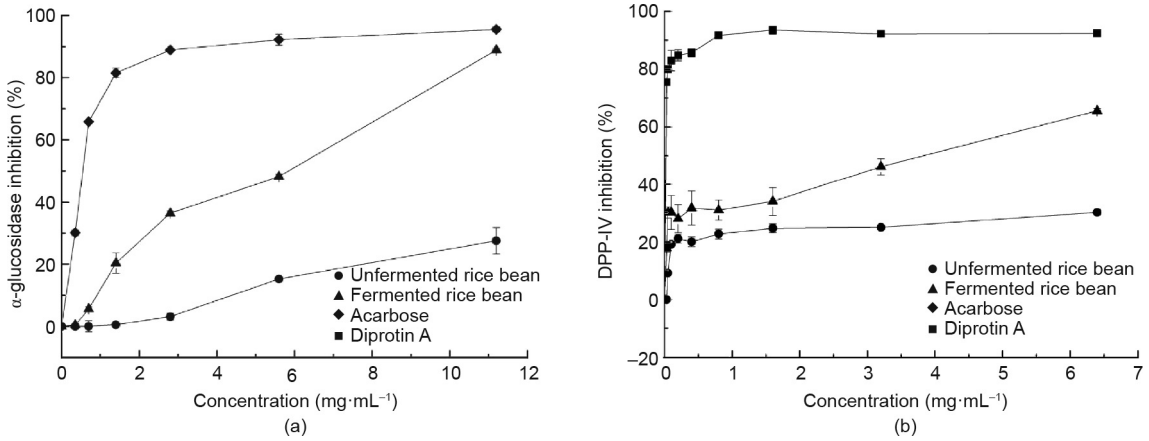

Fig. 3. (a) The α-glucosidase inhibitive activity and (b) DPP-IV inhibitive activity of unfermented and fermented rice beans with acarbose and diprotin A as the positive control, respectively. The sample concentrations in (a) and (b) were in the range of 0.35–11.2 mg·mL^{-1} and 0.03–6.4 mg·mL^{-1}, respectively.

beans increased by 1.6–2.2 times after *B. amyloliquefaciens* fermentation (Fig. 3(b)). Moreover, the fermented rice beans were found to be rich in peptides and total phenols (10.1 g per 100 g and 388.5 mg gallic acid per 100 g; Table 1). Most of the food-source-derived DPP-IV inhibitors reported in the literature are protein hydrolysates. In addition, flavonoids and phenolic acids have been reported to inhibit the DPP-IV enzyme activity [41]. Thus, fermented rice beans might be a potential source of DPP-IV inhibitors and functional food for the prevention of type-2 diabetes.

3.3.4. Anticoagulant activity

The anticoagulant activity of the aqueous extracts of unfermented and fermented rice beans was evaluated (Fig. 4). The aqueous extracts of the fermented rice beans inhibited the formation of fibrin clots in a dose-dependent manner (from 14.6% to 98.2%). The aqueous extracts of the unfermented beans exhibited lower anticoagulant activity (0%–6.3%) than the rice beans after fermentation. Fermented rice beans could suppress coagulation by inhibiting fibrinogenesis and thrombin activities. As blood clotting is an important factor in causing thrombotic diseases, the development of safe and cheap anticoagulant foods has attracted a great deal of attention [42]. Recent data has indicated that the hydrolysis product of peanut proteins by the alkaline protease showed a high anticoagulant activity of 95% at 40 mg·mL^{-1} [26]. Chickpeas fermented by *B. amyloliquefaciens* possess an anticoagulant activity of 80% at 1 mg·mL^{-1} [6], which is lower than that of heparin sodium. In this study, the anticoagulant activity of the rice beans fermented by *B. amyloliquefaciens* (98.2% at 1 mg·mL^{-1}; Fig. 4) was significantly higher than that of heparin sodium (57.7% at 1 mg·mL^{-1}; Fig. 4). To the best of our knowledge, this is the first report on the anticoagulant activity of rice beans fermented by *B. amyloliquefaciens*. In clinical practice, heparin sodium, warfarin, and aspirin are commonly used as anticoagulant agents. However, these drugs have safety issues, as they carry the adverse risk of major hemorrhage [43]. The natto kinase isolated from fermented soybeans has been shown to exhibit antithrombotic properties [44], and was determined to be safe after animal testing and human clinical trials [6,9]. As the water extracts of fermented rice beans have antithrombotic (Fig. 4) and thrombolytic effects (Fig. 1), they might be beneficial for the prevention of thrombotic diseases as dietary food supplements.

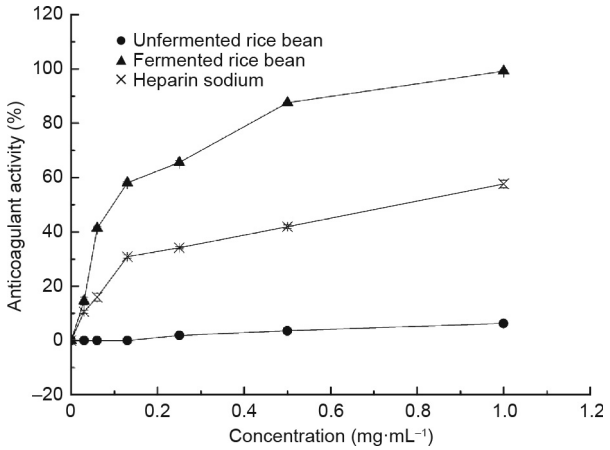

Fig. 4. Anticoagulant activity of unfermented and fermented rice beans. Heparin sodium was used as the positive control. The sample concentrations were in the range of 0.03–1.0 mg·mL^{-1}.

4. Conclusions

B. amyloliquefaciens was utilized as the starter culture for the fermentation of rice beans. Fermentation significantly improved the physicochemical properties of rice beans. The antioxidant, DPP-IV inhibitory, and α-glucosidase inhibitory activities of fermented rice beans were much higher than those of unfermented rice beans. Moreover, the anticoagulant activity of the fermented rice beans was greatly improved. The findings gained in this study indicate that rice beans fermented by *B. amyloliquefaciens* show potential as functional foods.

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Compliance with ethics guidelines

Shan Wu, Shuai Lu, Jun Liu, Shaoqing Yang, Qiaojuan Yan, and Zhengqiang Jiang declare that they have no conflict of interest or financial conflicts to disclose.

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