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Nutritional Improvement of Germinated Riceberry Rice (*Oryza sativa*) Cultivated with *Pleurotus ostreatus* Mycelium

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Abstract

Riceberry rice is a whole grain rice variety, which is rich in vitamin E, anthocyanin, phenolic acids, flavonoids, phytosterol antioxidants and fiber. The nutritional of Riceberry rice could be further improved by germination process, with this process the γ-aminobutyric acid (GABA) in rice samples is increased. Furthermore, solid stage fermentation (SSF) of rice and grains with fungi have been employed in the food industry such as rice fermented with *Aspergillus oryzae* for rice *koji* production, or soybean fermented with *Rhizopus oryzae* to produce Tempeh. This fermentation step is not only for the food preservation but also improve the nutritional in foods. In this study, the germination and fermentation with *Pleurotus ostreatus* mycelium can improve the nutritional content present in Riceberry rice. The amounts of GABA, anthocyanin, and bioactive substances contained in Riceberry rice (RR), germinated Riceberry rice (GR) and germinated Riceberry rice with *P. ostreatus* mycelium (MR) were compared. During germination, the γ-aminobutyric acid (GABA) content of RR increased by 71 and 73 % when further fermented with *P. ostreatus* mycelium. The highest level of GABA was found in the MR (38.58±0.29 mg/100 g) treatment. Furthermore, an untargeted metabolomics approach using Liquid Chromatograph Quadrupole Time-of-Flight Mass Spectrometer; LC-QTOF, revealed an abundance of various amino acids such as valine, alanine, histidine, methionine, L-glutamic acid as well as essential fatty acids such as steric acid and linoleic acid in germinated Riceberry rice with *P. ostreatus* mycelium samples. The results from this study could lead to nutritional improvement of Riceberry rice through fermentation with *P. ostreatus* mycelium, which could be applied in further rice flour applications and the product could be recognized as functional food.

Keywords: γ-aminobutyric acid, Anthocyanin, GABA, Germinated Riceberry rice, LC-QTOF, Metabolites profile, *Pleurotus ostreatus* mycelium

Introduction

Rice (*Oryza sativa* L.) constitutes an essential food source for more than half of the world's population. The black-purple variety Riceberry rice (RR) is a cross between the 2 Thai strains Khao Dawk Mali 105 (Thai Jasmine rice) and Hom-Nil (Thai non-glutinous purple rice), which has become one of the most highly consumed colored varieties in Thailand, being introduced in the year 2002 [1]. Riceberry rice is rich in various compounds such as vitamin E, anthocyanin, γ-aminobutyric acid (GABA), phenolic acids, flavonoids, phytosterol and antioxidant compounds, which could be beneficial to human health [2,3]. Recent studies have demonstrated the presence of several biological activities including anti-cancer, antioxidant, anti-hyperlipidemic, anti-hyperglycemic, and anti-inflammatory. These could be due to the abundance of compounds like peonidin-3-glucoside (P3G) and cyanidin-3-glucoside (C3G), which are glycoside forms of anthocyanin in the rice [4].

Germination process in rice is a bioconversion process that improves the organoleptic quality and nutritional availability in different cereal products. During germination, the L-glutamic acid is enzymatically decarboxylated by the glutamate de-carboxylase enzyme and yields γ-aminobutyric acid or GABA [5,6]. GABA is a neurotransmitter with anti-anxiety effects and is also a blood pressure regulator [7]. In animal and clinical studies, germinated rice showed anti-hyperlipidemic, anti-hypertensive, and anti-hyperglycemic activities, as well as a decrease in the risk of some chronic disorders such as cancer, diabetes, cardiovascular disease, and Alzheimer's disease [8]. Aside from the germinated rice, the essential amino acids, beta- glucan as well as GABA content can also be found in several edible mushrooms, such as

Agaricus bisporus, *Sparassis latifolia* and *Pleurotus* species. Of the latter, *Pleurotus ostreatus* is the most widely cultivated species of oyster mushroom [9-11].

Traditionally, fermentation is the simplest method to improve the nutritional content of foods. Both bacteria and fungi have been used in fermentation of food consumed by humans and animals. Nevertheless, fungi have been used at the 1st step of fermentation of rice grains and beans due to their ability to digest cellulose and starch [12]. Several studies have reported the beneficial effect of rice fermented with filamentous fungi, such as *Aspergillus oryzae* or mushroom mycelium including *Pleurotus ostreatus*. Specifically, these fungi not only digest the cellulose and increase the fiber and protein contents of the substrates, but they also improve the nutritional content, metabolite production and anti-aging properties of the rice grains [13,14]. Hence, improvement in nutritional and active ingredients could add more value to rice consumption.

In a previous study, the prebiotic properties of Riceberry rice (RR), germinated Riceberry rice (GR) and germinated Riceberry rice with *P. ostreatus* mycelium (MR) were reported. Specifically, fermentation of germinated Riceberry rice using the basidiomycete *Pleurotus ostreatus* mycelium for 3 days showed prebiotic activity score at the same level as inulin, which is used as the main prebiotic in many commercial products [15]. Recently, the trend of consumption of nutritious food, especially functional foods has been rising in the market. Hence, the present study sought to understand the chemical composition of Riceberry rice and how it changes during germination and fermentation with fungal mycelium. Therefore, the GABA content, anthocyanin content and the metabolite profiles of Riceberry rice (RR), germinated Riceberry rice (GR) and germinated Riceberry rice with *P. ostreatus* mycelium (MR) were determined using LC-MS and Q-TOF LC/MS technique. The data from this study could be used to support the bio convergence for the future development of Riceberry rice as a functional food.

Materials and methods

Materials

Riceberry rice (*Oryza sativa* L.) was obtained from a farmer group in Wiang Chai district, Chiang Rai province, Thailand. The mycelium of Oyster mushroom (*Pleurotus ostreatus*) was obtained from a local mushroom farm in Chiang Rai, Thailand. Analytical grade gamma-aminobutyric acid (GABA) and DPPH (2,2-Diphenyl-1-picrylhydrazyl) were bought from Sigma-Aldrich Pvt. Ltd, Singapore. Anthocyanin (Peonidin 3-glucoside chloride) was purchased from Phyto Lab. All media used for the cultivation of mushroom were bought from HiMedia Laboratories, LLC.

Sample preparation and germination method

The experiment including the germinated Riceberry rice (GR) was performed by following the previous method described by Soodpakdee *et al*. [15]. In brief, Riceberry rice (RR) was rinsed with sterile distilled water and soaked in plastic containers covered with colanders at 25 °C for 12 h. Three cycles of these actions were performed before discontinuing the germination process at 36 h. The germinated Riceberry rice (GR) was dried in a hot air oven (Memmert/une-500), which was set at 60 °C temperature for 24 h to stop the enzyme activity responsible for germination. Potato dextrose agar (PDA) was used to cultivate *Pleurotus ostreatus* for 15 days. The mycelium was then transferred into a flask containing 300 mL of potato dextrose broth (PDB) and incubated in a dark room for about 8 days. The germinated Riceberry rice with *P. ostreatus* mycelium (MR) was produced by transferring 10 % of the mycelium inoculum on 50 g (wet weight) of sterilized germinated Riceberry rice (GR). The fermentation procedure took place for 3 days at a temperature of 25 °C in a dark room. Treated Riceberry rice samples (germinated Riceberry rice, GR and Riceberry rice with *P. ostreatus* mycelium, MR) were dried in a hot air oven, which was set at 60 °C for 24 h. Samples were then ground into flour using the Hammer mill model CMC-20. The milled flour samples were then sifted with a series of 5 selected ASTM (American Standard Test Sieve Series) stainless-steel mesh sieves (No. 30, 35, 60, 120 and 325), corresponding to sieve opening dimensions of 600, 500, 250, 125 and 45 μm, respectively). The final flour particle size of 45 μm was used in all of the following experiments. The samples were stored in an amber zipped lock bag and kept in the desiccator at ambient temperature prior to further experiments.

Extraction and determination of GABA content in Riceberry rice samples using liquid chromatography mass spectrometry triple quadrupole

The extraction method for GABA (gamma-aminobutyric acid) and metabolites from the samples was adapted from the previous work conducted by Gökmen *et al.* [16]. From each rice flour powder, 1 g was extracted using deionized (DI) water at a 1:15 w/v ratio. The solutions were boiled in a water bath

(MemmertWNE-22) maintained at 90 °C for 5 min and centrifuged at 8,000 rpm for 15 min at room temperature using a Hettich/Universal 320R centrifuge. After centrifugation, the supernatant was diluted with 50 % (v/v) acetonitrile and filtered through a nylon filter (0.45 µm) prior to further analysis. The extracted samples were used for GABA and other metabolomics analysis. Each Riceberry rice sample was extracted with 3 replicates.

The protocol for GABA content analysis was done according to the procedure described by Soodpakdee *et al.* (2022) [15]. The range of GABA standard (Sigma-Aldrich) was provided at 0 to 100 ppm to create a standard curve. The GABA content in both standard compounds and extracted Riceberry rice samples were measured using the Liquid Chromatography Mass Spectrometry Triple Quadrupole system (Shimadzu, Nexera X2/LCMS 8060) with Infinity Lab Poroshell 120 HILIC-Z column (2.1×100 mm, 2.7 µm). Injection volume was taken as 1 µL, with 400 µL/min flowrate at 30 °C. The LCMS analyses of standards and samples were used as a linear gradient system of mobile phase A 0.1 % formic acid in DI water and B 10 mM ammonium formate in acetonitrile as follows: 3 min for 6 % solvent B, 4 min for 27 % solvent B, held at 27 % for 1min, then to 37 % for 1 min, and back to 0 % in 1.5 min, re-equilibrated column for 8.5 min at 0 % B (the initial conditions).

Extraction and determination of anthocyanin content in Riceberry rice samples

The anthocyanin content in extracted rice samples including RR, GR and MR were determined using a previous method from Poosri *et al.* [3]. The range of cyanidin and peonidin (Sigma-Aldrich) was provided at 0 to 500 ppm to create a standard curve. Five grams of each Riceberry rice samples were soaked in 15 mL of 1 % HCl in methanol (acidic methanol), and extracted consecutively by shaking at 200 rpm for 16 h at room temperature in dark condition. The solutions were filtrated through Whatman filter paper grade 1 and evaporated by Buchi R-100 rotary evaporator (Rotavap®). Extracted samples were dissolved with 2 % HCl in methanol to a final concentration of 1 mg/mL, which was then filtrated by using a syringe fitted with a 0.45 µm pore size nylon membrane filter. UltraHigh performance liquid chromatography (UHPLC) (Waters Acquity Arc model) was used for identification and quantification of anthocyanins present.

The extracted samples were analyzed using UHPLC following the working condition from Settapramote *et al.* [17]. A linear gradient system of mobile phase A (10 % formic acid in DI water) and mobile phase B (methanol) was constructed as follows: 5 % solvent B for 1 min (0 - 1 min), 27 % B for 14 min (1 - 15 min), 27 % B held for 1 min, then to 65 % B for 1 min (16 - 18 min), and back to 0 % B for 5 min (18 - 23 min). At the end, the column was re-equilibrated for 7 min (23 - 30 min) using the initial conditions (0 % B) with flow rate at 300 µL/min at 30 °C and injection volume was 1 µL using C_{18} column, detection at 515 nm. The samples loop was 25 µL, which were quantified by a reverse-phase UHPLC method at 515 nm and cyanidin 3-glucoside and peonidin3-glucoside were used to generate standard curves. All experiments were conducted in 3 replicates.

Analysis of metabolites in rice samples using LC-QTOF

Analysis of metabolites was modified from the procedure of Tyagi *et al.* [18]. Rice samples were analyzed by Liquid Chromatograph Quadrupole Time-of-Flight Mass Spectrometer; LC-QTOF (Agilent 1290 infinity II/G6545B QTOF/MS) analytical column used was Poroshell EC-C18 2.1×150 mm, 2.7 µm with flow rate at 400 µL/min at 35 °C. The LC-MS analyses of sample used a linear gradient system of mobile phase A (0.1 % formic acid in DI water) and mobile phase B (acetonitrile) as follows: 5 % solvent B for 1 min (0 - 1 min), 17 % B for 9 min (1 - 10 min), 17 % B held for 3 min, then to 100 % B for 7 min (13 - 20 min), 100 % B held for 5 min and back to 0 % B for 2 min (25 - 27 min). The column was re-equilibrated for 6 min (27 - 33 min) using the initial conditions (0 % B). The MS experiments were performed on an Agilent 6545 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer. Positive (ESI+) ion mode was used to conduct the mass spectrometric analyses. The parameters were set as follows: Capillary temperature at 300 °C, voltage at 500 V, spray voltage at 3.5 kV, tube lens voltage at 120 V and sheath gas flow at 12 arb. Fourier transform mass spectrometry (FTMS) full scan ion mode was employed with a resolution of 30,000 full width at half maximum (FWHM) and a mass scan range of 120 - 1,000 m/z. Centroid mode was used to acquire the spectra.

Compound identification was achieved by using molecular mass and emprirical formular finders databases "MassBank" (https://massbank.eu/MassBank/) and "The Human Metabolome Database (HMDB)" (https://hmdb.ca/). Multivariate statistical analyses including main component analysis (PCA) and heat maps were generated using MetaboAnalyst 5.0 program (https://dev.metaboanalyst.ca/home.xhtml). PCA was used to compare the changes of compounds among Riceberry rice samples, heat maps were drawn using peak areas of compounds by MetaboAnalyst 5.0 program.

Statistical analysis

All the data were recorded in triplicates and are presented in mean ± SD. The IBM SPSS Statistics Program Version 20 (SPSS, Inc., Chicago, IL, USA) was used for the data analysis. Data on GABA analysis was analyzed using One-way analysis of variance (ANOVA). When differences were found, Tukey HSD was initiated for multiple comparisons. Moreover, data on anthocyanin content was analyzed using independent student t-test. A *p*-value of < 0.05 was considered statistically significant.

Results and discussion

Metabolomics study in Riceberry rice samples

GABA Content

GABA is an inhibitory neurotransmitter that occurs naturally, and constitutes as a promising compound in neuroprotective treatments [19]. In this study, GABA concentrations in the 3 rice treatment samples (RR, GR, and MR) have been determined. The results showed that the germination process significantly improved GABA content in the Riceberry rice (**Figure 1**). Furthermore, the fermentation of *P. ostreatus* mycelium on germinated Riceberry rice showed the highest GABA concentrations (38.58±0.29 mg/100 g rice sample). This could be due to fungi and mushrooms including *P. ostreatus* having the enzyme for activating glutamate decarboxylase (GAD) and subsequent formation of GABA from L-glutamic acid, hence leading to increased GABA concentration in the MR treatment sample [20,21].

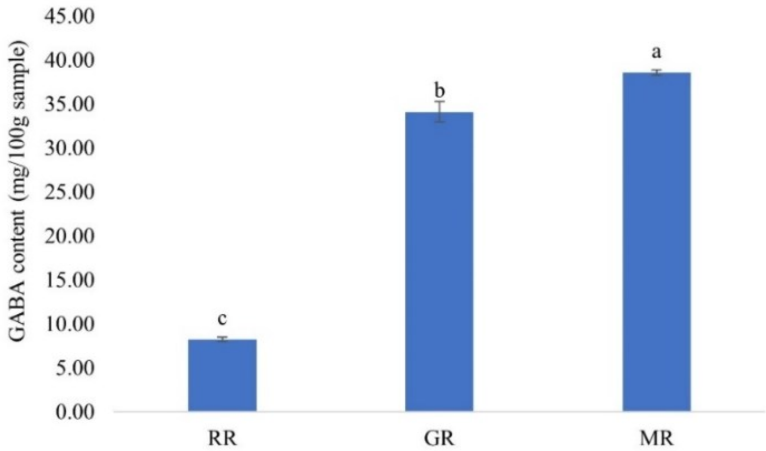

Figure 1 Comparison of GABA content among Riceberry rice (RR), Germinated rice (GR) and Germinated rice with mycelium (MR). Superscript letters ($^{a–c}$) denote significant differences (Tukey test, $p < 0.05$).

Anthocyanin content

Cyanidin and peonidin are the 2 main anthocyanin compounds found in Riceberry rice [22]. In this study, their concentrations are reported in **Table 1**. The RR treatment had the highest content of cyanidin and peonidin at 8.63±0.45 and 4.43±0.003 mg/100 g, respectively. It has been reported that black rice contains a higher concentration of phenolic compounds and anthocyanins than white rice [17]. Nevertheless, the cyanidin and peonidin content in the GR sample dramatically decreased to 0.14±0.72 and 0.11±0.005 mg/100 g, respectively and was not detected in the MR samples. This decrease could be due to the water soluble, heat intolerant nature of anthocyanins [22]. The germination process in this study, involved soaking and washing the rice with water for 3 cycles, which could lead to the loss of anthocyanins from Riceberry rice. Furthermore, in the MR sample the germinated Riceberry rice sample was cooked using sterilization, which could explain the absence of the cyanidin and peonidin. These results are in agreement with those of Zaupa *et al.* [23], who reported that the antioxidant, and polyphenolic compounds including anthocyanin were reduced during domestic cooking process.

Table 1 Cyanidin and peonidin content in rice sample.

Compounds In 100 g of dried rice (mg)	Samples		*p*-value
	RR	GR	
Cyanidin	8.63±0.45	0.14±0.72	0.001
Peonidin	4.43±0.003	0.11±0.005	0.011

Noted: RR: Riceberry rice; GR: germinated Riceberry rice

Amino acids content

Essential amino acid levels were measured in RR, GR and MR treatments (**Figure 2(A)**). The highest amino acid level was observed in the MR sample. This could be due to either internal enzymatic breakdown of non-functional proteins in Riceberry rice and upregulated amino acid biosynthesis during germination [24] and/or due to external enzymatic breakdown by the protease enzymes from fungi during fermentation. The hydrolytic activity of this enzyme has been reported from *Aspergillus* spp. and *Trichoderma harzianum* during solid stage fermentation [25,26]. Both processes could lead to digestion of proteins in the Riceberry rice into short chain amino acids. Alanine, Cholylglycines, Gamma-glutamate, Histidine, His-Asn-Cys, Leucyl-aspartyl-val, L-cystiene, L-leucine, L-glutamic acid, L-glutamine, Lysine butyrate, Methionine, Phenylalanine, Trp-Ser-Ser, Valine, and Avl-Ile-Cys, all of which are necessary amino acids were significantly increased in MR sample, whereas L-Homoserine, L-leucine and Pro-val-Leu were not present in MR (**Figure 2(A)**). These results are in agreement with previous findings by Tyagi *et al.* [18]. The authors reported the increase in amino acid level in geminated brown rice in comparison with non-germinated brown rice due to the activation of dormant enzymes in the rice grains. On the other hand, germinated Riceberry rice (GR) contained 2 types of amino acids including L-glutamine and L-glutamic acid. This finding is in support of the activity of enzyme glutamate decarboxylase producing γ-Aminobutyric acid (GABA) from L-glutamic acid during the germination process of brown rice [5] and correlation among the amino acids present in the samples can be seen by using Principal component analysis (PCA). When comparing PC1 with PC2, the 3 samples were divergent and separate. After comparing PC1 with PC3, the GR sample profile was related to the MR sample, but the RR sample grouped separately (**Figure 2(B)**). The results of PCA analysis confirmed the pattern found in the heat map analysis.

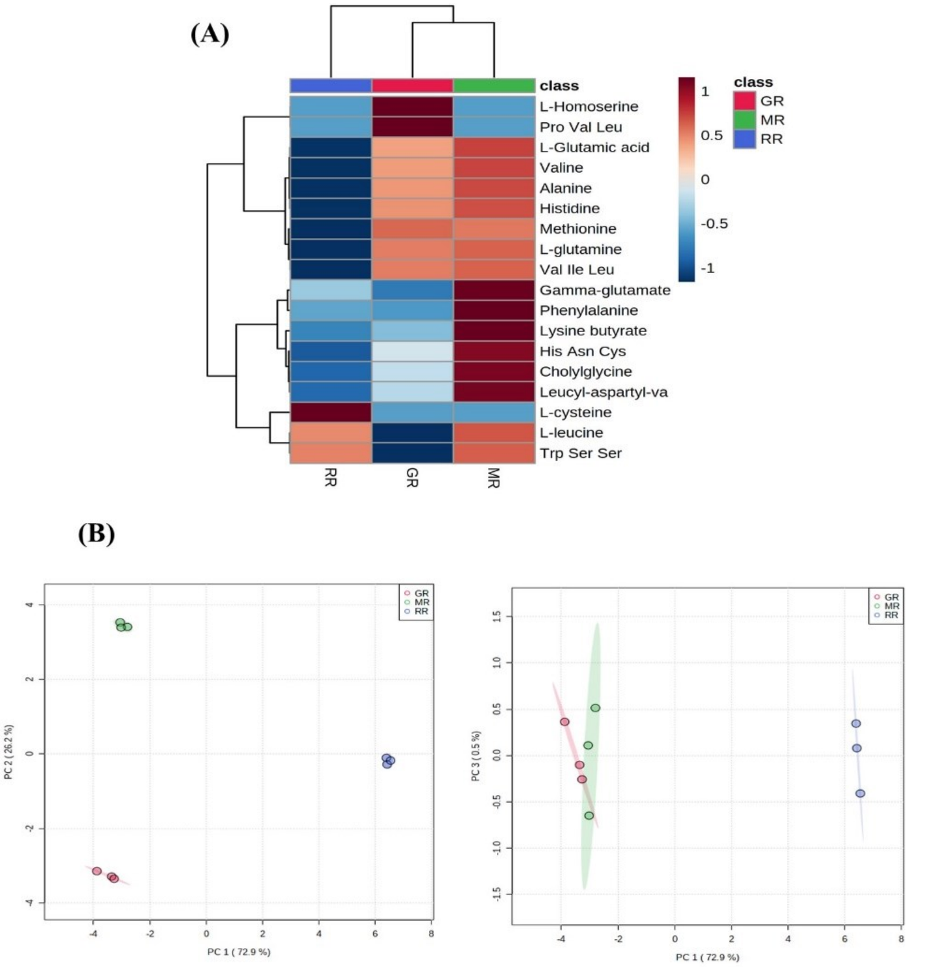

Figure 2 Amino acid levels in Riceberry rice (RR), Germinated Riceberry rice (GR) and Germinated Riceberry rice with mycelium (MR) (A) Different levels of amino acids in samples from blue to red; blue shade indicates the lowest abundance in samples, while red shade denotes the highest amount. (B) Principal component analysis (PCA) of RR, GR and MR was performed by comparing PC1 with PC2 and PC1 with PC3.

Fatty acid content

Fatty acids are necessary components of living organisms producing energy and essential elements that are necessary to cells and subcellular organelles [27]. In total, 6 fatty acids were found in the samples at different levels (**Figure 3(A)**). The highest level was present in the Riceberry rice sample (RR). Linoleic acid, octadecadienoic acid and stearic acid are generally the types of fatty acids, which can be found in RR [28]. During the germination process, stearic acid, glycerol and linoleic acid were decreased possibly due to lipid breakdown in relation to energy production. These results match those of Kim *et al.* [8], who reported decreased levels of glycerol, stearic acid and palmitic acid during germination. In this study, several fatty acids were increased after fermentation with *P. ostreatus* mycelium. This could be due to biosynthesis of fatty acids and phospholipids in the fungal biomass [29]. This result is in agreement with a previous study by Rousta *et al.* [30]. The authors reported that the short chain omega-3 α-linolenic acid (ALA) and omega-6 linoleic acid (LA) increased from 0.6 to 8.4 and from 21.7 to 68.4 (mg/g dry weight sample), respectively in the oat flour, when fermented with *Aspergillus oryzae*. Particularly, fermentation has been suggested as a strategy to improve the nutritional content of meals, both in terms of enhanced bioavailability of nutrients and the creation of end products that are beneficial to health [31]. Moreover, principal component analysis (PCA) also showed that the fatty acid levels in the 3 samples separated (**Figure 3(B)**).

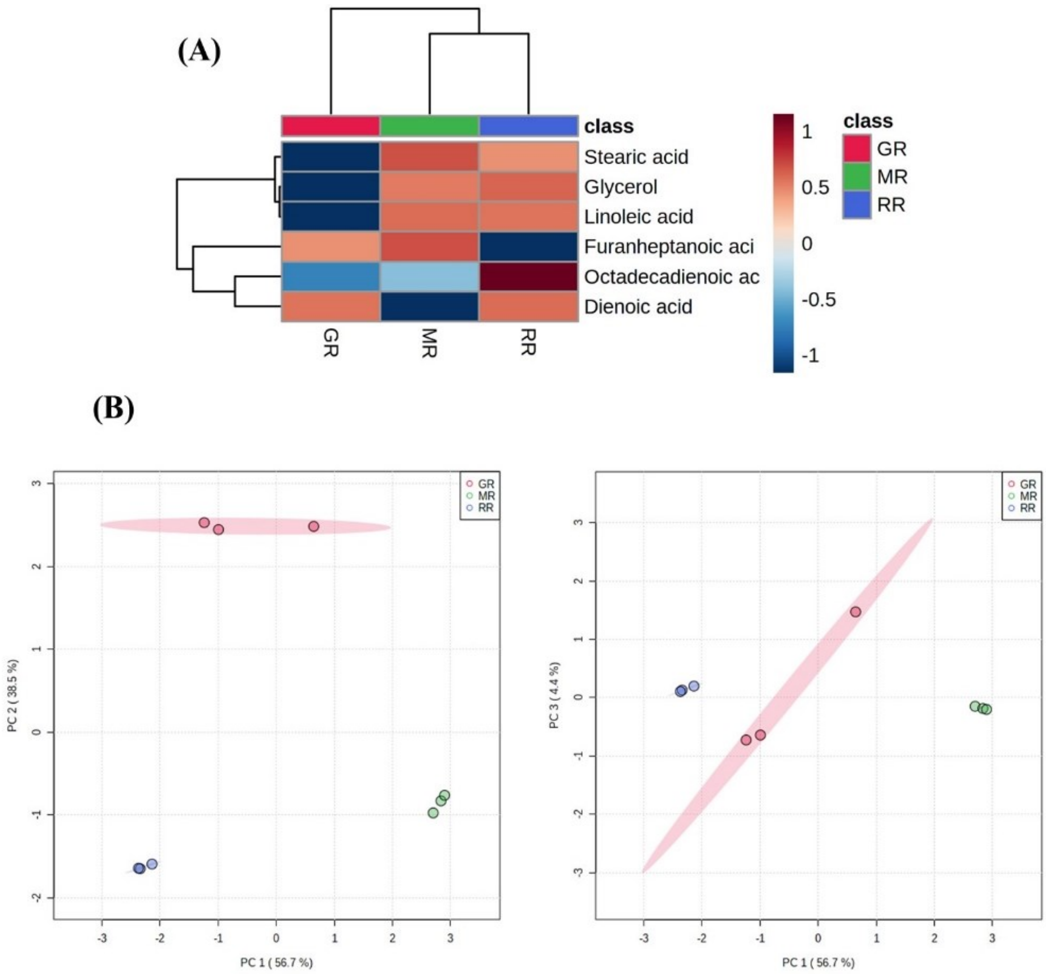

Figure 3 Fatty acid levels in Riceberry rice (RR), Germinated Riceberry rice (GR) and Germinated Riceberry rice with mycelium (MR) (A) Different levels of fatty acid in samples from blue to red; blue shade indicates the lowest abundance in samples, while red shade denotes the highest amount. (B) Principal component analysis (PCA) of RR, GR and MR was performed by comparing PC1 with PC2 and PC1 with PC3.

Phenolic compound content

Phenolic constituents are classified as antioxidants, which are important in reducing the risk of several types of diseases, especially chronic food-related diseases [32]. Results of phenolic content in different samples are shown in **Figure 4(A)**. The highest level of phenolic compounds was shown in the RR sample, while levels of phenolic compound in GR and MR were decreased. The results from the heat map were consistent with the anthocyanin content reported in anthocyanin content section. Phenolic compounds might be degraded during the preparation process of the MR sample, when heat is applied. Principal component analysis (PCA) showed that the RR sample was divergent form both the GR and MR samples (**Figure 4(B)**).

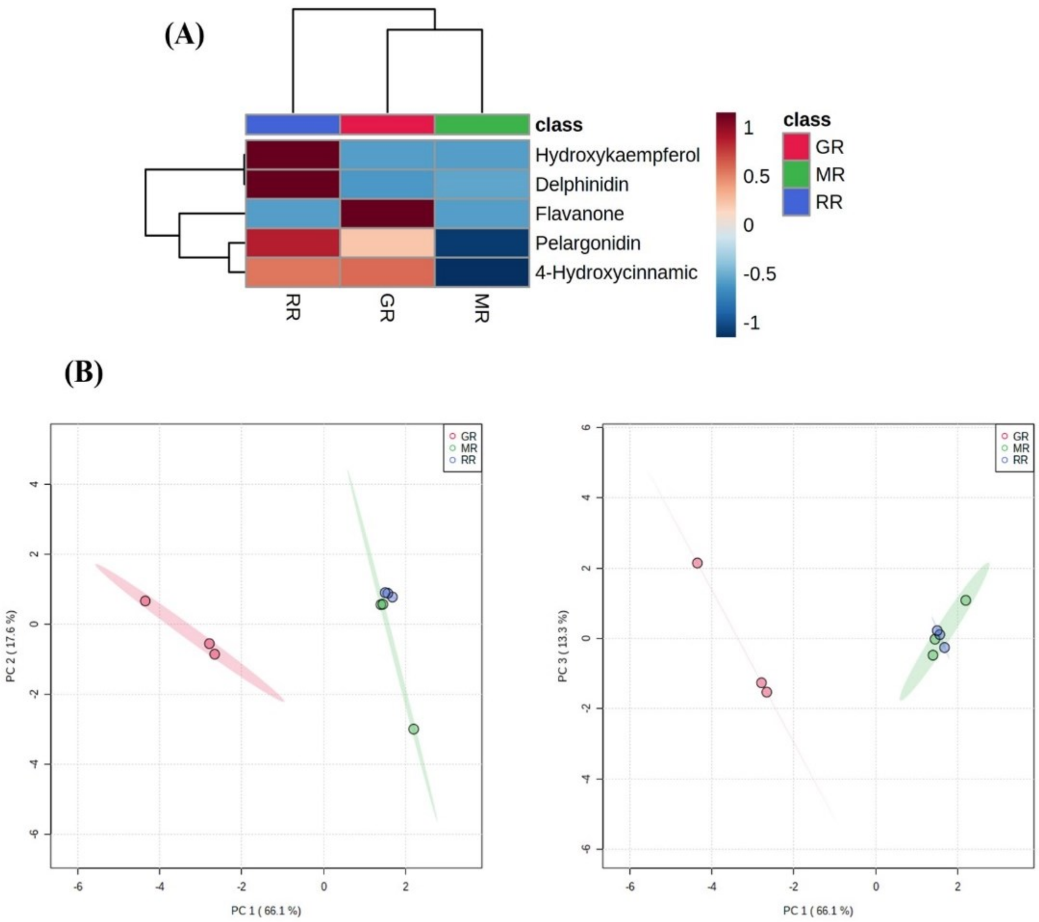

Figure 4 Phenolic compounds levels in Riceberry rice (RR), Germinated Riceberry rice (GR) and Germinated Riceberry rice with mycelium (MR) (A) Different levels of phenolic compounds in samples from blue to red; blue shade indicates the lowest abundance in samples, while red shade denotes the highest amount. (B) Principal component analysis (PCA) of RR, GR and MR was performed by comparing PC1 with PC2 and PC1 with PC3.

Conclusions

In our experiment, we found that Riceberry rice fermented with *Pleurotus ostreatus* mycelium (MR) exhibited high GABA content, amino acids, and fatty acids, when compared to the other samples, germinated Riceberry rice (GR) and Riceberry rice (RR), respectively. The variations in the metabolite profiles during germination were examined using LC-QTOF, and differences in metabolite profiles were displayed using heat map and Principal Component Analysis (PCA). The results clearly supported the decarboxylation mechanism of L-glutamic acid in GABA synthesis during Riceberry rice germination and enzymatic breakdown of the protein in rice grains by fungal enzymes. Herein, a short-term fermentation process showed nutritional improvement of Riceberry rice (MR) compared to normal (RR) and germinated

(GR) samples. In this study, around 29 different metabolites including amino acids, fatty acids and phenolic compounds were identified. The 3 treatments had completely different metabolic profiles. Nevertheless, the phenolic compounds in Riceberry rice were not improved neither during the germination process nor during fermentation with *P. ostreatus* process. This could be due to the light sensitive and heat intolerant nature of these compounds. Moreover, these findings suggest that the Riceberry rice fermented with Pleurotus ostreatus mycelium sample is a promising material for the development of rice as a functional food given the high GABA, amino acid, and essential fatty acid contents. Due to the prebiotic properties and the increase in small peptide molecules released after fermentation, an *in-vitro* on anti-colorectal cancer study should be conducted in the future to further confirm the health promoting effects and functional food development of the treated and untreated Riceberry rice samples.

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