Bioscience, Biotechnology, and Biochemistry, 2024, **88**, 1117–1125
https://doi.org/10.1093/bbb/zbae082
Advance access publication date: 22 June 2024
Regular Paper

Solid-state cultivation of multiple industrial strains of *koji* mold on different Thai unpolished rice cultivars: biotransformation of phenolic compounds and their effects on antioxidant activity

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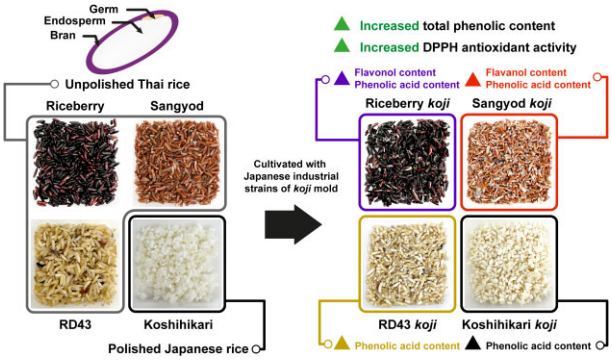
Abstract

Colored rice is abundant in polyphenols, and *koji* molds have potential for biotransformation. This study aimed to produce Thai-colored rice *koji* to study its polyphenolic biotransformation. Four industrial *koji* mold strains: *Aspergillus oryzae* 6001, *A. oryzae* 6020, *A. sojae* 7009, and *A. luchuensis* 8035, were cultivated on unpolished Thai-colored rice (Riceberry and Sangyod), unpolished Thai white rice (RD43), and polished Japanese white rice (Koshihikari). We discovered that *koji* molds grew on all the rice varieties. Methanol extracts of all rice *koji*s exhibited an approximately 2-fold or greater increase in total phenolic content and DPPH antioxidant activity compared to those of steamed rice. Moreover, quercetin, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, ferulic acid, caffeic acid, protocatechuic acid, vanillic acid, (+)-catechin, and (–)-epicatechin content increased in Riceberry and Sangyod *koji* samples. Consequently, *Aspergillus* solid-state cultivation on unpolished Thai-colored rice exhibited higher functionalization than the cultivation of unpolished Thai white rice and polished Japanese white rice.

Keywords: antioxidant activity, *koji* mold, polyphenols, solid-state fermentation, Thai colored rice

Graphical abstract



Biotransformation of polyphenolic compounds and their effects on antioxidant activity in unpolished Thai and polished Japanese rice *koji* samples.

Recently, there has been an increasing interest in exploring biological sources such as plant materials and microorganisms for the development of products in the food, cosmetic, and pharmaceutical industries.

Microbial conversion of certain secondary metabolites not only involves structural modifications to specific functional groups without altering the overall structural components but may also lead to enhanced biological activities and physical properties. For instance, compactin, a fungal metabolite produced by *Penicillium citrinum*, undergoes hydroxylation mediated by *Streptomyces carbophilus*, resulting in the formation of pravastatin, which has been used in the treatment of hyperlipidemia (Endo, Kuroda and Tsujita 1976; Matsuoka *et al.* 1989). We have previously reported that a novel cyclic dipeptide oxidase from *Streptomyces albulus*, KO23, catalyzes the conversion of (–)-phenylahistin, a fungal metabolite produced by *Aspergillus ustus*, into dehydropheny-

Received: 5 April 2024; **Accepted:** 3 June 2024

lahistin. This metabolite exhibited a thousand-fold increase in potent inhibitory activity against cell division compared with (–)-phenylahistin (Kanzaki *et al.* 2002). Subsequently, dehydrophenylahistin was further converted to plinabulin, which is currently in Phase III trials with the aim of increasing the survival of cancer patients (Tonra *et al.* 2020). We also reported that aldehyde reductase derived from Baker's yeast converts the aldehyde group of oleuropein aglycone, a secondary metabolite found in olive leaf ethanol extracts, to a hydroxymethyl group, resulting in increased antioxidant activity (Kanzaki and Nitoda 2008). This microbial reduction compound is commercially available as a cosmetic product named "B-Olivol." These results highlight the potential of microorganisms to transform the secondary metabolites produced by plant materials or microorganisms, which may lead to enhanced functionalization.

Solid-state cultivation is a method for growing microorganisms on solid materials, which results in the production of specific metabolites generated by enzymes during microbial growth. For example, we previously reported that pochonicine, a fungal metabolite of *Pochonia suchlasporia* var. *suchlasporia* TAMA 87, produced during solid-state cultivation of rolled barley, potently inhibits chitin-degrading β-*N*-acetylglucosaminidases (GlcNAcases) (Usuki *et al.* 2009). Solid-state cultivation has been widely employed to produce fermented foods, including *koji* molds, and has long been used in food applications (Yamashita 2021). Through extensive breeding and maintenance efforts by Japanese meisters to ensure the absence of toxin production (Machida *et al.* 2005; Yamada *et al.* 2016), *koji* molds have been designated as "Generally Recognized as Safe (GRAS)" by the Food and Drug Administration (Hamajima *et al.* 2016) and have been used for the cultivation of rice, wheat, and soybean through solid-state cultivation to yield a variety of edible products, including *sake* (Japanese rice wine), *honkaku shochu* (Japanese distilled liquor), *shoyu* (soy sauce), *miso* (fermented soybean paste), *mirin* (sweet sake for seasoning), and *amazake* (Japanese non-alcoholic sweet beverage) (Yamashita 2021).

Rice (*Oryza sativa* L.), particularly the white rice variety, is a common staple mostly consumed in Asia. Unpolished rice, including brown and colored rice varieties, not only contains typical nutrients such as proteins and carbohydrates, as well as white rice, but is also enriched in secondary metabolites. In addition, colored rice varieties in Thailand have been reported to have abundant secondary metabolites. Riceberry (*Oryza sativa* L. ssp. *indica* cv. Riceberry), a newly developed rice variety developed in 2002 at Kasetsart University, Thailand, features black-colored rice grains that are rich in anthocyanins, such as cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside (Settapramote *et al.* 2018; Poosri *et al.* 2019; Wongsa *et al.* 2019). Another Thai-colored rice, Sangyod (*Oryza sativa* L. ssp. *indica* cv. Sangyod Phatthalung), is notable for its richness in flavanols and proanthocyanidins, resulting in red-colored rice grains (Thitipramote *et al.* 2022). Furthermore, unpolished Thai-colored rice varieties were enriched in other secondary metabolites, such as flavonols, phenolic acids, and γ-oryzanol (Limtrakul, Semmarath and Mapoung 2019).

In Japan, for over a thousand years, polished rice, where the bran layer, as a source of secondary metabolites, is removed, leaving behind a substrate rich in carbohydrates and proteins, has been extensively used in the production of "rice *koji*." Consequently, the biotransformation of secondary metabolites in polished rice *koji* is less frequently observed. However, Shin *et al.* (2019) reported the cultivation of a single *koji* mold strain on colored rice bran obtained from a single rice variety with the aim of investigating changes in total phenolic content (TPC), antioxidant activity, and polyphenolic composition. We hypothesized that using different rice cultivars, which may contain various types and contents of polyphenols, for cultivation with different strains of *koji* mold could result in diverse metabolic biotransformations, especially concerning polyphenolic compounds, thereby affecting the antioxidant activity. Therefore, conducting a study utilizing 4 different whole-grain rice cultivars to cultivate with 4 industrial strains of *koji* mold for *koji* cultivation could provide insights into these varied microbial biotransformations.

Here, we aimed to cultivate industrial strains of *koji* mold on Thai-colored rice cultivars, namely Riceberry and Sangyod, to investigate their potential for biotransformation, resulting in the creation of highly functional materials distinct from their raw materials. We employed 4 industrial strains of *koji* mold: *A. oryzae* 6001, *A. oryzae* 6020, *A. sojae* 7009, and *A. luchuensis* 8035, each recognized for their distinctive characteristics in the expression of carbohydrate-hydrolyzing and proteolytic enzymes. Furthermore, we cultivated RD43, an unpolished Thai white rice variety, with the same *koji* molds to produce RD43 *koji*, facilitating a comparative analysis of the change in secondary metabolites within unpolished Thai rice, representative of Indica rice. In parallel, we employed polished Japanese rice, representing Japonica rice, from the Koshihikari rice variety to produce Koshihikari *koji*, serving as the traditional rice *koji* for comparison. To achieve this objective, we endeavored to perform the microbial transformation of secondary metabolites in these materials through *Aspergillus* solid-state cultivation to produce highly functional materials.

Materials and methods

Chemicals and reagents

The standard compounds included cyanidin-3-*O*-glucoside (Tokiwa Phytochemical, Chiba, Japan); caffeic acid, (+)-catechin hydrate, gallic acid, protocatechuic acid, quercetin dihydrate, and vanillic acid (Nacalai Tesque, Kyoto, Japan); (–)-epicatechin (Tokyo Chemical Industry, Tokyo, Japan); ferulic acid, cycloartenyl ferulate, γ-oryzanol, and quercetin-3-*O*-glucoside (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (EMD Millipore Corp., MA, USA).

Plant materials and microorganisms

Riceberry (*Oryza sativa* L. ssp. *indica* cv. Riceberry; Thai black rice), Sangyod (*Oryza sativa* L. ssp. *indica* cv. Sangyod Phatthalung; Thai red rice), and RD43 (*Oryza sativa* L. ssp. *indica* cv. RD43; Thai white rice) were purchased from a local farm in Phatthalung, Thailand. Koshihikari (*Oryza sativa* L. ssp. *japonica* cv. Koshihikari; Japanese white rice) was commercially available. Four industrial strains of *koji* mold, consisting of *A. oryzae* 6001 (yellow *koji* mold), *A. oryzae* 6020 (yellow *koji* mold white mutant strain), *A. sojae* 7009 (yellow *koji* mold for soy sauce production), and *A. luchuensis* 8035 (black *koji* mold), were preserved at Higuchi Matsunosuke Shoten Co., Ltd.

Production of rice *koji* samples

Samples of rice *koji* were prepared using the conventional method for making rice *koji*, as described by Yamashita (2021). In brief, 4 distinct rice varieties, including 3 unpolished Thai rice varieties (Riceberry, Sangyod, and RD43) and a polished Japanese rice variety from Koshihikari (approximately 89% rice polishing ratio), were washed with tap water before soaking and incubating at

30 °C for 3 h. The soaked rice was steamed for 50 min, cooled, and inoculated with various strains of industrial *koji* mold. Uninoculated steamed rice derived from each rice cultivar served as the control. The fermentation process began at 30 °C for 20 h, followed by slight mixing, and continuous incubation at 32 °C for 3 h and 35 °C for 2 h. The mixture was remixed and covered with filter paper before being incubated at 35 °C for 18 h to produce rice *koji*, with a total cultivation time of 43 h. Both steamed rice and rice *koji* samples were stored at −25 °C in a freezer until extraction with methanol.

Methanol extraction of steamed rice and rice *koji* samples

Samples of steamed rice and rice *koji* were processed using an electric grinder (Model CG-8316, HadinEEon, Shenzhen, China) to obtain ground material. One gram of the ground sample was weighed, and its moisture content was determined using an Infrared Moisture Analyzer (model MA35, Sartorius Mechatronics, Osaka, Japan). Subsequently, 100 mg of the ground sample was extracted with 1 mL methanol for 24 h. This mixture was then filtered through defatted cotton and adjusted to a volume of 1 mL using a volumetric flask before being stored at −25 °C for subsequent analysis of the TPC, antioxidant activity, and UHPLC-PDA-MS.

Determination of total phenolic content and antioxidant activity

TPC was assessed using the method described by Singleton and Rossi (1965) and expressed as gallic acid equivalent. Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method, which was modified from Takebayashi, Tai and Yamamoto (2002) and expressed as Trolox equivalent. Total phenolic content and antioxidant activity were calculated with the moisture content (Table S1) and reported as the gram dry weight of steamed rice and rice *koji* samples.

UHPLC-PDA-MS analysis of the methanol extract in steamed rice and rice *koji* samples

UHPLC-PDA-MS analysis of the methanol extract of steamed rice and rice *koji* samples was conducted using a modified method described by Lei *et al.* (2019). In brief, the ACQUITY UPLC H-Class System (Waters Corp., Milford, MA, USA) was equipped with the ACQUITY UPLC® BEH C18 1.7 µm 2.1 × 50 mm Column (Waters Corp., Wexford, Ireland) and the ACQUITY UPLC® BEH C18 1.7 µm VanGuard™ Pre-Column 2.1 × 5 mm (Waters Corp., Wexford, Ireland). The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B), with a gradient of 95% A (v/v) at 0 min, 69% A (v/v) at 12 min, 0% A (v/v) at 15–19 min, and 95% B at 24–29 min, with a column temperature of 60 °C and a flow rate of 0.56 mL/min. The methanol extract of steamed rice and rice *koji* samples was injected at 0.2 µL through the column and detected using an ACQUITY photodiode array (PDA) eλ detector (Waters Corp., Milford, MA, USA) with a scanning range of 200–600 nm and an ACQUITY QDa detector (Waters Corp., Milford, MA, USA) with electrospray ionization. The capillary voltage was set at 0.8 kV, and the cone voltage was set at 15 V for both positive and negative charge modules. The scanning range for mass detection was *m/z* 100.00–800.00. Empower 3 software database version 7.20.00.00 (Waters Corp., Milford, MA, USA) was used for the acquisition and data processing. Compounds: protocatechuic acid (**1**), (+)-catechin (**4**), cyanidin-3-*O*-glucoside (**5**), caffeic acid (**6**), vanillic acid (**7**), (−)-epicatechin (**8**), ferulic acid (**9**), quercetin-3-*O*-glucoside (**10**), and quercetin (**12**), were identified by comparing their retention times, UV spectra, and MS spectra in the extracts with those in the authentic standards (Figure S1). The quantities of these compounds were determined using calibration curves from authentic standards. Additionally, proanthocyanidin dimer (**2**), proanthocyanidin trimer (**3**), and isorhamnetin-3-*O*-glucoside (**11**) were identified by MS spectra. Their peak areas were measured at the maximum absorption wavelengths and quantified using calibration curves from structural analogs, expressed as (+)-catechin equivalent for proanthocyanidins (**2, 3**) and quercetin-3-*O*-glucoside equivalent for isorhamnetin-3-*O*-glucoside (**11**). One of the γ-oryzanols, cycloartenyl ferulate (**13**), was identified by comparing its retention time, UV spectrum, and MS spectrum in the extracts with those in the authentic standard (Figure S2). The other 3 γ-oryzanols, 24-methylenecycloartanyl ferulate (**14**), campesteryl ferulate (**15**), and β-sitosteryl ferulate (**16**), were identified by comparing their UV spectra and MS spectra in the extracts with those in cycloartenyl ferulate. These γ-oryzanols were quantified using a cycloartenyl ferulate (**13**) calibration curve and expressed as its equivalent. The content of each compound was calculated with the moisture content (Table S1) and reported as the gram dry weight of steamed rice and rice *koji* samples.

Statistical analysis

Data obtained from triplicate measurements are presented as mean ± standard deviation (SD). One-way ANOVA was conducted using the R program (version 4.2.0) with Tukey's Honestly Significant Difference test to compare the steamed rice and rice *koji* samples for each parameter. Differences were considered significant when the *P* values were less than .05 ($P < .05$).

Results and discussion

Growth of four *koji* mold strains on Riceberry, Sangyod, RD43, and Koshihikari

Four industrial strains of *koji* mold were able to grow on 3 unpolished Thai rice cultivars as the cultivated materials. The growth of *koji* mold on these materials appeared to be almost similar to that observed on Koshihikari, a polished Japanese rice variety typically used for rice *koji* cultivation, as visually observed (Figure 1). Notably, all rice *koji* samples cultivated with *A. oryzae* 6001, *A. oryzae* 6020, and *A. luchuensis* 8035 displayed a whitish coloration of the rice grains due to hyphal growth, while those with *A. sojae* 7009 exhibited a yellowish coloration. The amylase and protease activity levels, used as indicators of *koji* mold growth, were consistent across all rice *koji* samples (data not shown), confirming the growth of *koji* mold on unpolished Thai rice grains.

Traditionally, in Japan, rice *koji* has been made using polished Japanese white rice as the cultivated material. Based on our experience, the cultivation of *koji* mold on unpolished Japanese rice (Japonica rice) exhibited slow growth, as observed visually. However, in this study, the cultivation of *koji* mold on unpolished Thai rice grains (Indica rice) was almost comparable to the growth observed on polished Japanese rice grains. This suggests that the difference in rice cultivars between Indica and Japonica rice may influence *koji* mold growth. Since *koji* mold grew on unpolished Thai-colored rice, unpolished Thai white rice, and polished Japanese white rice, it is possible to observe the microbial biotransformation of polyphenolic compounds due to *koji* mold activity. Therefore, polyphenol content and antioxidant activity were investigated.

Figure 1. Four steamed rice and sixteen rice *koji* samples, namely Riceberry *koji*, Sangyod *koji*, RD43 *koji*, and Koshihikari *koji* cultivated with *A. oryzae* 6001, *A. oryzae* 6020, *A. sojae* 7009, and *A. luchuensis* 8035.

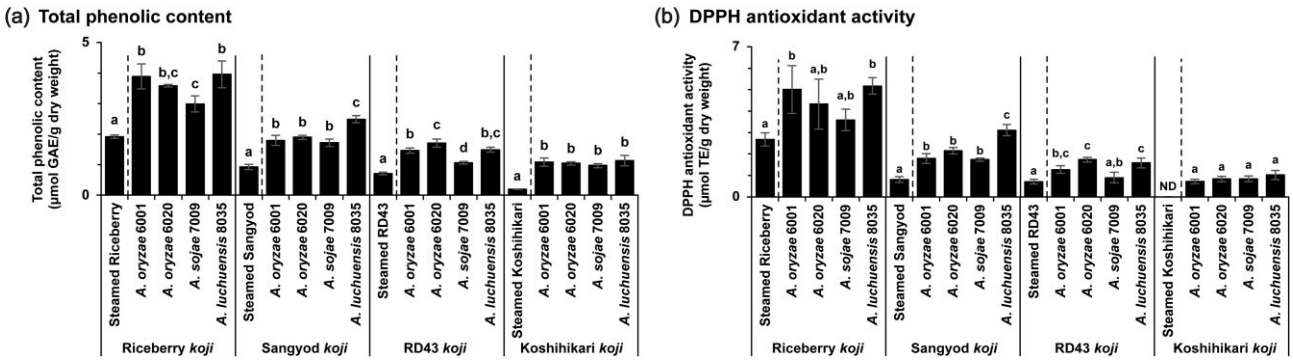


Figure 2. Total phenolic content (a), expressed as μmol gallic acid equivalent (GAE)/g dry weight, and DPPH antioxidant activity (b), expressed as μmol Trolox equivalent (TE)/g dry weight, in the extracts of steamed rice and rice *koji* samples. Lowercase letters indicate a significant difference ($P < .05$) by Tukey's range test within each rice variety.

Total phenolic content and DPPH antioxidant activity

After *koji* mold cultivation, the TPC (Figure 2a and Table S2) in the extracts of all the rice *koji* samples significantly increased by 2–6 times compared to that of steamed rice. It is possible that *koji* mold has the ability to increase the content of alcohol-soluble polyphenols from soluble and insoluble polyphenols in rice grains through microbial biotransformation. The increase in alcohol-soluble polyphenol content may be attributed to the growth of *koji* mold on the rice grains.

Rocchetti *et al.* (2022) reported in the review article that polyphenols can be found in both free and bound forms, and the bound polyphenols can be determined after alkaline hydrolysis, which reacts on, such as ester bond, and acid hydrolysis, which reacts on the ester and glycosidic bonds between bound polyphenols and cell wall-like materials. Additionally, Yamashita (2021) reported that *koji* mold has the ability to solid-state cultivation-specifically express various types of enzymes, such as esterases, glycoside hydrolases, proteases, and lipases. Based on the above facts, we believe that the increase in polyphenol content in the *koji* methanol extracts was possibly due to enzymatic reactions by *koji* mold, which released polyphenols from their precursors; in other words, these enzymes possibly converted bound polyphenols into their free form.

Given the variations in polyphenol composition among the rice varieties, we cultivated 4 distinct rice cultivars with 4 different industrial strains of *koji* mold. The TPC after cultivation exhibited variations depending on the rice cultivars used as well as the *koji* mold strains used. This suggests that microbial biotransformation, leading to an increase in alcohol-soluble polyphenols, may vary depending on the rice cultivar and *koji* mold strain used.

When considering the different rice varieties used, the TPC in the extracts of Riceberry *koji* exhibited the highest levels, representing 2–3 times higher than that of Sangyod *koji*, 2–4 times higher than that of RD43 *koji*, and 3–7 times higher than that of Koshihikari *koji*, which corresponds to the polyphenol content of the original materials. This suggests that the use of high-polyphenol-content materials as cultivation materials may be one of the factors in obtaining high alcohol-soluble polyphenol content after cultivation. Additionally, the higher TPC in the extracts of unpolished Thai rice *koji* than that of polished Japanese rice *koji* may be attributed to the presence or absence of the rice bran layer, which serves as the main source of polyphenols in rice grains (Pereira-Caro *et al.* 2013), although the differences in Thai and Japanese rice cultivars used in the cultivation also played a role. Moreover, the higher TPC in the extract of Riceberry *koji* compared to that of Sangyod *koji* and RD43 *koji* may be related to the different polyphenol contents in the original materials among these unpolished Thai rice varieties, especially the polyphenols present in the rice bran layer, resulting in variations in the content of alcohol-soluble polyphenols through microbial biotransformation. Furthermore, a significant increase in TPC was detected after cultivation in the extract of polished Japanese rice *koji* from the Koshihikari rice variety. This indicated that even after removing the rice bran layer, certain soluble polyphenol compounds in the rice endosperm were capable of becoming alcohol-soluble polyphenols through microbial biotransformation by *koji* mold activity.

When considering the different *koji* mold strains, the TPC in the extract of rice *koji* cultivated with *A. oryzae* 6001 was almost comparable to that of rice *koji* cultivated with *A. luchuensis* 8035. Moreover, the TPC in the extracts of rice *koji* cultivated with *A. oryzae* 6020 and *A. sojae* 7009 was slightly lower than that of rice *koji* cultivated with *A. oryzae* 6001 and *A. luchuensis* 8035. It is possible that the *koji* mold strain *A. luchuensis* 8035 has the potential to increase the content of alcohol-soluble polyphenols through microbial biotransformation from cultivated materials at a level comparable to that of *A. oryzae* 6001. In the *koji* mold strain *A. oryzae* 6020, the ability to increase the content of alcohol-soluble polyphenols was lower than the ability in the *koji* mold strain *A. oryzae* 6001, although both belong to the same species. Almost all the TPC in the extracts of rice *koji* mold strain *A. sojae* 7009 had the lowest content. This may be related to the fact that *A. sojae* may be more suitable for cultivation on soybean (Yamashita 2021). These observations show that the different rice cultivars and *koji* mold strains used in rice *koji* cultivation can induce different increases in the content of alcohol-soluble polyphenols through microbial biotransformation.

The DPPH antioxidant activity (Figure 2b and Table S2) in the extracts of all the rice *koji* samples significantly increased by 1.3–4 times compared to that of steamed rice. When considering the different rice varieties used, the DPPH antioxidant activity in the extracts of Riceberry *koji* exhibited the highest levels, representing 2–3 times higher than that of Sangyod *koji*, 3–4 times higher than that of RD43 *koji*, and 5–7 times higher than that of Koshihikari *koji*. When considering the different *koji* mold strains, the DPPH antioxidant activity in the extract of rice *koji* cultivated with *A. oryzae* 6001 was almost comparable to that of rice *koji* cultivated with *A. luchuensis* 8035. Moreover, the DPPH antioxidant activity in the extracts of rice *koji* cultivated with *A. oryzae* 6020 and *A. sojae* 7009 was slightly lower than that of rice *koji* cultivated with *A. oryzae* 6001 and *A. luchuensis* 8035. It was found that the DPPH antioxidant activity in the extracts of all rice *koji* samples exhibited a high correlation with TPC. Thus, it can be inferred that the DPPH antioxidant activity in the extracts of these rice *koji* samples can be largely explained by the polyphenol content.

When considering the extracts of Riceberry *koji*, which utilized whole rice grain for cultivation, the TPC and DPPH antioxidant activity in the extract after 43 h cultivation were significantly increased compared to those in the extracts of steamed Riceberry. These results contrasted with the TPC and DPPH antioxidant activity in the extract of Korean black rice bran cultivated with the *koji* mold strain *A. oryzae*, which exhibited a comparable level after 48 h cultivation and a significant increase after 96 h cultivation compared to those of steamed Korean black rice bran (Shin *et al.* 2019). It is speculated that *koji* mold activity in the microbial transformation of polyphenolic compounds to increase the content of alcohol-soluble polyphenols is faster when the cultivated material is supplied with typical nutrients, such as carbohydrates and proteins, as evidenced in the unpolished Thai-colored rice *koji*, supporting its highly functionalized property.

Since the content of alcohol-soluble polyphenol increased in the extracts of unpolished Thai and polished Japanese rice *koji* samples, it is possible that this increase may be attributed to polyphenol compounds supplied in the rice grains. Therefore, the polyphenol compounds in all rice *koji* samples were investigated using UHPLC-PDA-MS analysis.

Anthocyanin content

A thorough analysis of polyphenols and other compounds using UHPLC-PDA-MS revealed that anthocyanins, specifically cyanidin-3-*O*-glucoside (**5**) and peonidin-3-*O*-glucoside were not detected in any of the methanol extracts of steamed Riceberry and Riceberry *koji* samples (Figure S1), but these compounds were detected only in the methanol extract of Riceberry dry rice (data not shown) in this study. This is supported by the removal of pigments during the rice-soaking step for 3 h.

Flavonol contents

As shown in Figure 3, the content of quercetin-3-*O*-glucoside (**10**) in the extracts of Riceberry *koji* cultivated with *A. oryzae* 6001 and *A. luchuensis* 8035 increased by 1.5 times or more, while the content in the extracts of Riceberry *koji* cultivated with *A. oryzae* 6020 and *A. sojae* 7009 exhibited only a slight increase compared to that of steamed Riceberry. In contrast, the content of quercetin (**12**) in the extracts of Riceberry *koji* cultivated with *A. oryzae* 6020 and *A. sojae* 7009 increased by approximately 1.7 times, while this content in the extracts of Riceberry *koji* cultivated with the other strains showed only a slight increase compared to that of steamed Riceberry (Table S3). This phenomenon was also observed in the content of isorhamnetin-3-*O*-glucoside (**11**) in the extract of Riceberry *koji*, especially in the extract of Riceberry *koji* cultivated with *A. oryzae* 6001, representing an approximate 2-fold increase compared to that of steamed Riceberry, although the isorhamnetin content was not detected in any of the extract of rice *koji* samples. This suggests that an increase in the content of alcohol-soluble flavonols may be due to soluble flavonols in

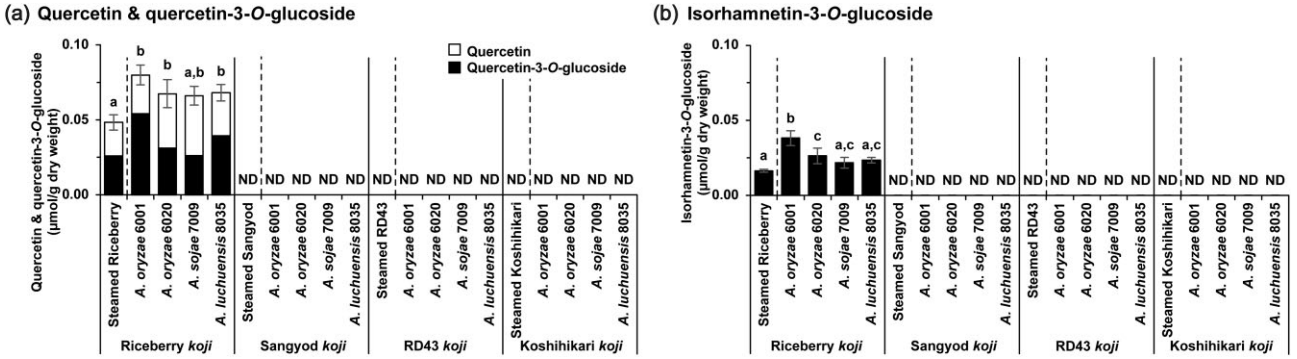


Figure 3. Flavonol content in the extracts of steamed rice and rice *koji* samples: (a) combined content of quercetin (**12**), and quercetin-3-*O*-glucoside (**10**), and (b) isorhamnetin-3-*O*-glucoside (**11**). Isorhamnetin-3-*O*-glucoside (**11**) content was expressed as quercetin-3-*O*-glucoside equivalent (Q3GE). Lowercase letters indicate a significant difference ($P < .05$) by Tukey's range test within each rice variety. "ND" stands for "not detected."

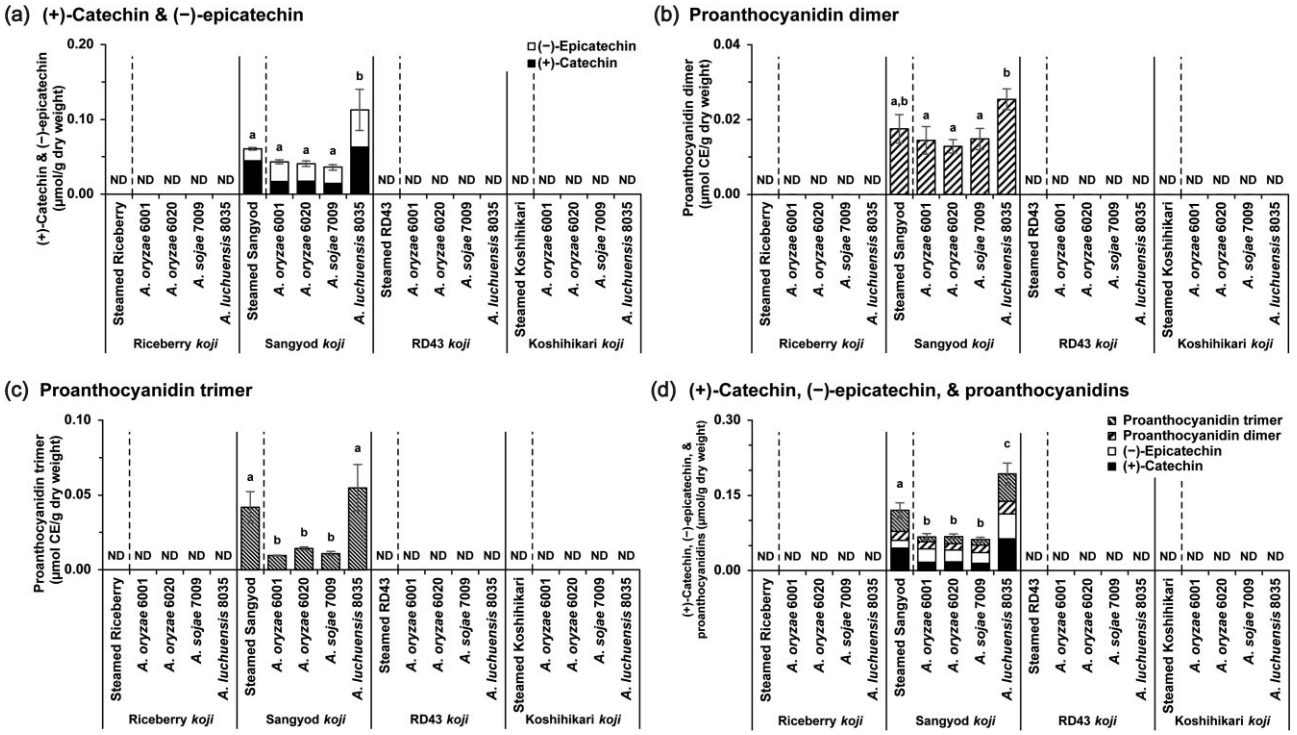


Figure 4. Flavanol and proanthocyanidin content in the extracts of the steamed rice and rice *koji* samples: (a) combined content of (+)-catechin (**4**), and (–)-epicatechin (**8**), (b) proanthocyanidin dimer (**2**), (c) proanthocyanidin trimer (**3**), and (d) combined content of flavanols (**4, 8**), and proanthocyanidins (**2, 3**). Proanthocyanidin content was expressed as (+)-catechin equivalent (CE). Lowercase letters indicate a significant difference ($P < .05$) by Tukey's range test within each rice variety. "ND" stands for "not detected."

Riceberry grain through microbial biotransformation by *koji* mold activity, as observed in the flavonol content of Riceberry *koji*. Furthermore, certain *koji* mold strains, particularly *A. oryzae* 6020 and *A. sojae* 7009, may convert quercetin glycosides in the form of soluble flavonols in rice grains into soluble aglycone forms, resulting in an increased alcohol-soluble flavonol content. Similarly, the biotransformation of isorhamnetin glycosides, as soluble flavonols in the rice grain, into their aglycone form was inferred.

Flavanol contents

Flavanols, consisting of (+)-catechin (**4**), (–)-epicatechin (**8**), proanthocyanidin dimer (**2**), and proanthocyanidin trimer (**3**) were detected only in steamed Sangyod and Sangyod *koji* samples (Figure 4). The content of proanthocyanidin dimer (**2**), and proanthocyanidin trimer (**3**) in the extract of Sangyod *koji* cultivated with *A. luchuensis* 8035 increased by approximately 1.5 times compared to that of steamed Sangyod (Figure 4b and c). This suggests that the *koji* mold strain *A. luchuensis* 8035 may have the potential to increase the content of alcohol-soluble proanthocyanidins in Sangyod rice grains through microbial biotransformation. However, these proanthocyanidins (**2, 3**) may not be depolymerized into their monomers, as observed from the high proanthocyanidin content after cultivation. Moreover, the combined content of (+)-catechin (**4**) and (–)-epicatechin (**8**) in the extract of Sangyod *koji* cultivated with *A. luchuensis* 8035 significantly increased by approximately 2 times compared to that of steamed Sangyod (Figure 4a and Table S4), suggesting an increase in the content of alcohol-soluble flavanols through microbial biotransformation, with no further conversion by *koji* mold activity.

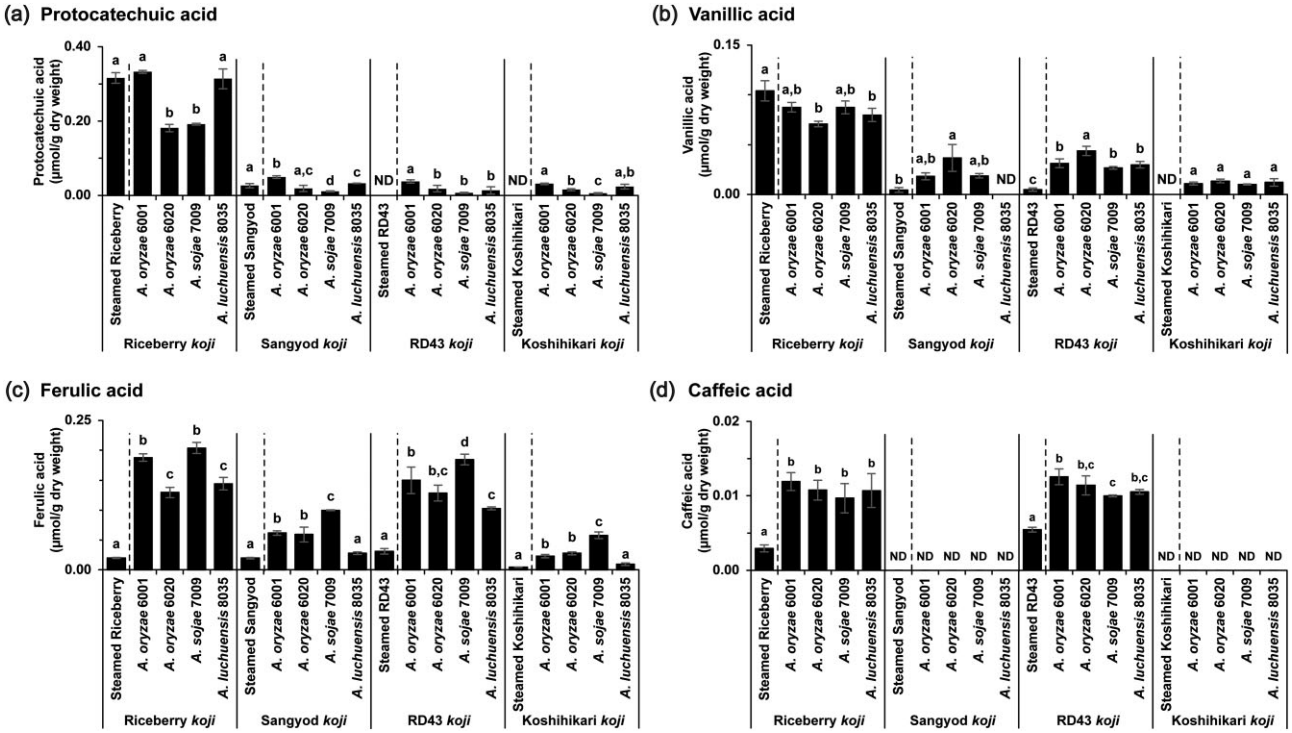


Figure 5. Phenolic acid content in the extracts of the steamed rice and rice *koji* samples: (a) protocatechuic acid (**1**), (b) vanillic acid (**7**), (c) ferulic acid (**9**), and (d) caffeic acid (**6**). Lowercase letters indicate a significant difference ($P < .05$) by Tukey's range test within each rice variety. "ND" stands for "not detected."

The content of proanthocyanidin dimer (**2**) in the extracts of Sangyod *koji* cultivated with *A. oryzae* 6001, *A. oryzae* 6020, and *A. sojae* 7009 slightly decreased, while the content of proanthocyanidin trimer (**3**) significantly decreased by approximately 4 times after cultivation (Figure 4b and c). This suggests that *koji* mold strains may have the potential to increase the content of alcohol-soluble proanthocyanidins in Sangyod rice grains through microbial biotransformation and depolymerization of proanthocyanidins into their monomers. The combined content of (+)-catechin (**4**) and (–)-epicatechin (**8**) in the extracts of Sangyod *koji* cultivated with *A. oryzae* 6001, *A. oryzae* 6020, and *A. sojae* 7009 decreased by one-third compared to that of steamed Sangyod (Figure 4a). This suggests that the depolymerized monomers may undergo further biotransformation through *koji* mold activity, as evidenced by the decreased levels of both components.

The total flavanol content (Figure 4d), consisting of (+)-catechin (**4**), (–)-epicatechin (**8**), proanthocyanidin dimer (**2**), and proanthocyanidin trimer (**3**) in the extract of Sangyod *koji* cultivated with *A. luchuensis* 8035 increased by approximately 1.5 times, whereas that of Sangyod *koji* cultivated with other strains decreased by approximately 2 times after cultivation. This supports the idea that changes in the flavanol content in the extracts of Sangyod *koji* varied depending on the *koji* mold strains.

Phenolic acid content

The content of ferulic acid (**9**) in the extract of Riceberry *koji* and RD43 *koji* was higher than that of Sangyod *koji* and Koshihikari *koji*, regardless of the *koji* mold strains (Figure 5c). Moreover, caffeic acid (**6**) was detected only in the extracts of Riceberry and RD43 steamed rice and rice *koji* samples (Figure 5d, Tables S3, and S5). Regardless of the *koji* mold strain, both the content of ferulic acid (**9**) and caffeic acid (**6**) increased by 5 times or more in the extracts of all rice *koji* samples and by 2 times or more in the extracts of Riceberry and RD43 *koji* samples after cultivation. With the reports on the release of ferulic acid (**9**) which is bound to the arabinoxylan structure (Schefer, Oest and Rohn 2021), by feruloyl esterase (Koseki *et al.* 2009), it is possible that the contents of alcohol-soluble ferulic acid may increase from those of insoluble ferulic acid. Additionally, ferulic acid (**9**) presented in the form of soluble phenolic acids in unpolished rice grain (Tian, Nakamura and Kayahara 2004), may contribute to the increase in the content of ferulic acid (**9**) in the extract through microbial biotransformation. When considering the differences in *koji* mold strains, the content of ferulic acid (**9**) in the extracts of Riceberry *koji* samples cultivated with *A. oryzae* 6001 and *A. sojae* 7009 was significantly higher than that of Riceberry *koji* samples cultivated with the other strains, suggesting the possibility of varying activity in the release of ferulic acid (**9**) depending on the *koji* mold strain.

The content of protocatechuic acid (**1**) and vanillic acid (**7**) in the extracts of steamed Riceberry was 14–20 times higher than those of steamed Sangyod, RD43, and Koshihikari rice cultivars (Figure 5a and b), reflecting the content in the original materials. After *koji* mold cultivation, the content of protocatechuic acid (**1**) in the extracts of Riceberry *koji* cultivated with *A. oryzae* 6020 and *A. sojae* 7009 decreased by approximately 1.7 times in both extracts. Moreover, the content of vanillic acid (**7**) in the extracts of Riceberry cultivated with *A. oryzae* 6020 and *A. luchuensis* 8035 decreased by approximately 1.4 times compared to that of steamed Riceberry. Moreover, the content of protocatechuic acid (**1**) and vanillic acid (**7**) in the extracts of Sangyod *koji* and RD43 *koji* mostly increased after cultivation and varied across different *koji* mold strains (Tables S4, S5 and S6). This suggests that *koji* mold may have the ability to increase alcohol-soluble protocatechuic acid and alcohol-soluble vanillic acid through microbial

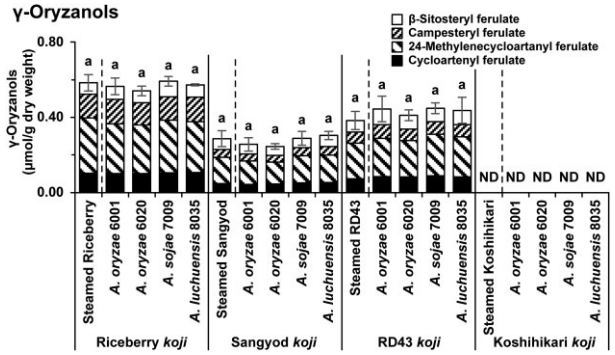


Figure 6. γ-Oryzanols content in the extracts of steamed rice and rice *koji* samples. γ-Oryzanols comprised cycloartenyl ferulate (**13**), 24-methylenecycloartanyl ferulate (**14**), campesteryl ferulate (**15**), and β-sitosteryl ferulate (**16**). The latter three γ-oryzanols were expressed as cycloartenyl ferulate equivalent (CFE). Lowercase letters indicate a significant difference ($P < .05$) by Tukey's range test within each rice variety. "ND" stands for "not detected."

biotransformation from both soluble protocatechuic acid and soluble vanillic acid in rice grains. Furthermore, some strains of *koji* mold may present activity in further biotransformations of alcohol-soluble protocatechuic acid and alcohol-soluble vanillic acid.

The content of protocatechuic acid (**1**), vanillic acid (**7**), and ferulic acid (**9**) in the extract of Koshihikari *koji* almost increased after *koji* mold cultivation and varied depending on the *koji* mold strains used. Moreover, the content of protocatechuic acid (**1**), vanillic acid (**7**), and ferulic acid (**9**) in the extracts of Koshihikari *koji* was lower than those of unpolished Thai rice *koji*. Given the presence of soluble phenolic acids in the rice endosperm (Tian, Nakamura and Kayahara 2004; Hashizume *et al.* 2014), it is possible that *koji* molds have the ability to increase the content of alcohol-soluble phenolic acids from the soluble phenolic acid in rice endosperm through microbial biotransformation, and this ability varied depending on the *koji* mold strains used.

3,4-dihydrophenylacetic acid and phloroglucinol are well-known microbial metabolites of quercetin produced by the intestinal bacteria (Braune *et al.* 2001). However, as these compounds were not detected in any methanol extract of rice *koji* samples in this study, it is suggested that the metabolic pathway in *koji* mold may be different from that in intestinal bacteria.

γ-Oryzanols content

γ-Oryzanols (**14-17**) were detected in the extracts of all unpolished Thai rice *koji* samples. Regardless of the *koji* mold strain, the content of γ-oryzanols (**14-17**) in the extract of Riceberry *koji* was approximately twice as high as that of Sangyod *koji* and approximately 1.5 times as high as that of RD43 *koji*. γ-Oryzanols (**14-17**) were not detected in any of the extracts of Koshihikari *koji*. When considering the results regarding ferulic acid (**9**) and γ-oryzanols (**14-17**), in which the structure of γ-oryzanols (**14-17**) consists of a mixture of ferulic acid esters of phytosterols and triterpene alcohols, the content of ferulic acid (**9**) increased in the extracts of all rice *koji* samples after cultivation. Despite this increase, the content in the extracts of these unpolished Thai rice *koji* samples was not significantly different before and after *koji* mold cultivation (Figure 6), suggesting that *koji* mold may not release ferulic acid (**9**) from the structure of γ-oryzanols (**14-17**) into a soluble form. It is plausible that the structure of γ-oryzanols (**14-17**) may remain intact, as it is almost insoluble in water, thus possibly avoiding enzymatic breakdown.

Conclusion

Four industrial strains of *koji* mold grew well on unpolished Thai-colored rice varieties, leading to an increase in alcohol-soluble polyphenols from soluble and insoluble polyphenols in rice grains through microbial biotransformation of *koji* mold activity. It is possible that *koji* mold has the potential to convert some soluble flavonol glycosides in rice grains into alcohol-soluble flavonol aglycones, as well as to release ferulic acid (**9**) and caffeic acid (**6**) from insoluble polyphenols in rice grains into alcohol-soluble compounds. Moreover, the increased alcohol-soluble polyphenol content influenced the increase in antioxidant activity. This indicates that *Aspergillus* solid-state cultivation on unpolished Thai-colored rice grain increased the bioavailability of polyphenols, supporting the potential of unpolished Thai-colored rice *koji* as a highly functionalized material.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and its online supplementary material.

Author contribution

H.K. and T.N. (Teruhiko Nitoda) designed the research; H.Y. and T.N. (Takuro Nakagawa) conducted experiment (*koji* mold cultivation); J.J. conducted experiment (polyphenol analysis); J.J. and H.K. analyzed the data; J.J. wrote the manuscript draft; H.K. supervised the manuscript preparation; H.K., T.N. (Teruhiko Nitoda), and H.Y. reviewed the manuscript.

Funding

This work was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) under Grant Number JP22H02242; and the Tojuro Iijima Foundation for Food Science and Technology.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgments

We would like to thank Editage (www.editage.jp) for English language editing. We are grateful to Ms. Emi Uchida for assisting with the experimental analysis.

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Received: 5 April 2024. **Accepted:** 3 June 2024