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Changes in Flavor- and Aroma-Related Fermentation Metabolites and Antioxidant Activity of Glutinous Rice Wine Supplemented with Chinese Chestnut (*Castanea mollissima* Blume)

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Abstract: Traditional glutinous rice wine (TGRW) has been fermented in China for over 9000 years. Recently, an innovative regional variation of TGRW, chestnut rice wine, banli mijiu (BLMJ), was developed by adding Chinese chestnut (*Castanea mollissima* Blume) into the fermentation brew. The objective of this study was to characterize the effects of chestnut on the nutritional, aromatic, and antioxidant properties of TGRW. To compare the aromatic sensory profiles between TGRW and BLMJ, the free amino acids and ethyl carbamate, phenolic, and flavonoid contents were determined. In addition, the antioxidant properties, including reducing power, metal chelation, and free radical scavenging activities, were also compared. A total of 98 distinct flavor components were identified in BLMJ, among which 38 were detected by sniffing instrument, compared to 77 distinct flavor components in TGRW. BLMJ thus contains a wider range of flavor components, but similar alcohol, acid and reducing sugar profiles compared with TGRW. Twenty-five free amino acids were detected in both wines, with lower contents of each in BLMJ compared with TGRW. BLMJ also exhibited stronger antioxidant properties than TGRW. The findings of this study suggest that chestnut can increase the diversity of aromatic components and improve antioxidant qualities of traditional rice wine.

Keywords: chestnut; rice wine; amino acid; antioxidant activity; phenols; sensory components

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1. Introduction

Castanea, in *Fagaceae*, contains four species of cultivated chestnut, including Chinese chestnut (*C. mollissima* Blume), Japanese chestnut (*C. crenata* Sieb. et Zucc.), European chestnut (*C. sativa* Miller), and American chestnut (*C. dentata*). Among these, *C. mollissima* Blume, one of only a few starch-containing nuts also rich in sugars, vitamins, and trace elements, has been previously explored as a complementary ingredient in different foods due to its nutritional and "warm" flavor properties [1]. Research has shown that the proteins in Chinese chestnuts have a nutritionally balanced amino acid composition and amino acid score (AAS) that meet the essential amino acid requirements recommended for adult nutrition by the FAO/WHO (2013) [2]. In addition, chestnuts also contain numerous polyphenols such as gallic acid and ellagic acid, flavonoids (rutin, quercetin, and apigenin), and tannins [3]. Chestnuts and chestnut by-products are used as a source of natural antioxidant food additives to extend shelf-life. For example, the extracts of chestnut by-products were shown to

decrease protein oxidation and increase lipid stability in beef patties [4]. In addition to the value placed on chestnut in traditional medicinal treatments [5], studies of neonatal calves have shown that the chestnut tannin, proanthocyanidin, acts as an anti-diarrhea agent [6], as well as an anti-gastritis agent in humans [7] and broiler chickens [8]. Furthermore, extracts of chestnut tree, nut, and shell have all been shown to exhibit cytotoxicity against human colon cancer cells and adenocarcinoma cells [9].

Rice wine, known as sake in Japan, takju in Korea, and huangjiu or mijiu in China, is traditionally consumed throughout East Asia, Southeast Asia, and Northeast India. It is produced by fermentation of rice starch that has been converted to sugars through microbial enzymatic activity. Alcoholic, fermented rice wines have a long history in China dating back to ca. 7000 BC [10], since fermentation has been widely used to preserve foods and potentially enhance their nutritional value [11]. Due to its perceived pharmacological, nutritional, and sensory benefits, mijiu rice wine has thus played a major role in the development of Chinese culture, and persistent demand has driven horticultural advances, intensification of agriculture, and innovations in its processing techniques [12,13].

Since raw ingredients may retain their biological activity throughout the rice wine fermentation process, a growing list of nutrient-rich raw materials have been explored for their ability to add value to rice wine [14]. Among these materials, chestnut has emerged as an ideal additive to Chinese rice wine fermentation because of its high starch content, which ensures a final ethanol content comparable with traditional rice wines, and because it contains protein levels that are sufficient to support microbial growth during fermentation [15–17]. Although the process technology [16,17] and specific yeast strains effective for Chinese chestnut wine fermentation have been studied [13], the effects of chestnut on rice wine flavor profile and nutrient content remain unknown. In light of its potential increasing usage, it therefore is important to identify the flavor compounds and nutrients found in BLMJ but not in TGRW. In this current study, the flavor profile and chemical characteristics of BLMJ attributable to chestnut were explored through analysis of flavor-related and phenolic compounds, as well as by quantifying its antioxidant activity and free amino acid contents.

2. Materials and Methods

The glutinous rice was obtained from Jilin Longyuan Rice Industry Co., Ltd. Chinese chestnut (*C. mollissima* Blume) was obtained from QianXi of Hebei province of China. DPPH (1,1-diphenyl-2-bitryl group, purity $\geq$ 98%), BHT (butylated hydroxytoluene, purity $\geq$ 98%), and tocopherol (purity $\geq$ 96%) were obtained from Sigma-Aldrich Co. (Shanghai, China). The other reagents used in this study were obtained from Aladdin Co. (Shanghai, China). This jiuqu starter was obtained from the Beizong Rice Wine Co., Ltd. (Zhangjiakou, China), and the active dry yeast was obtained from the Angle Yeast Co., Ltd. (Yichang, Hubei, China).

2.1. Brewing Process of Chestnut Rice Wine

The brewing process of BLMJ was performed in four stages, according to the methods described by Zou and coworkers (Figure 1) [16,18]. In Stage I, the raw material treatment stage, the chestnuts are peeled and washed, then chopped by a pulverizer into particles similar in size to grains of rice. The glutinous rice is then washed and soaked for 12 h at 20 °C, after which the water is drained. The chestnut particles and soaked glutinous rice are then mixed in a 1:3 (*w*/*w*) ratio. In Stage II, the incubation stage, the rice/chestnut mixture is steamed for 35 min, then cooled to 35 °C by spraying with water. Afterwards, 4% (*w*/*w*) jiuqu and 0.2% (*w*/*w*) active dry *Saccharomyces cerevisiae* (Angle Yeast Co., Ltd. of China) are added, which is specially used for rice wine fermentation. Jiuqu is a fermentation starter culture for rice wine consisting of a mixture of microorganisms such as *Aspergillus oryzae*, *Rhizopus oryzae*, and *S. fibuligera*, similar to Koji used in Japanese sake production. This jiuqu starter was obtained from the Beizong Rice Wine Co., Ltd. and is typically used for rice wine production in Northern China. The microbial community in this jiuqu is different

from that used for Shaoxing yellow rice wine fermentation, and has been described by Ren and colleagues [19]. The main cultured bacterial strains in Beizong jiuqu starter were *Bacillus*, and the main fungal strains were identified as *Aspergillus oryzae*, *Aspergillus niger*, *Talaromyces radicus*, *Neurospora crassa*, and *Absidia corymbifera* [19].

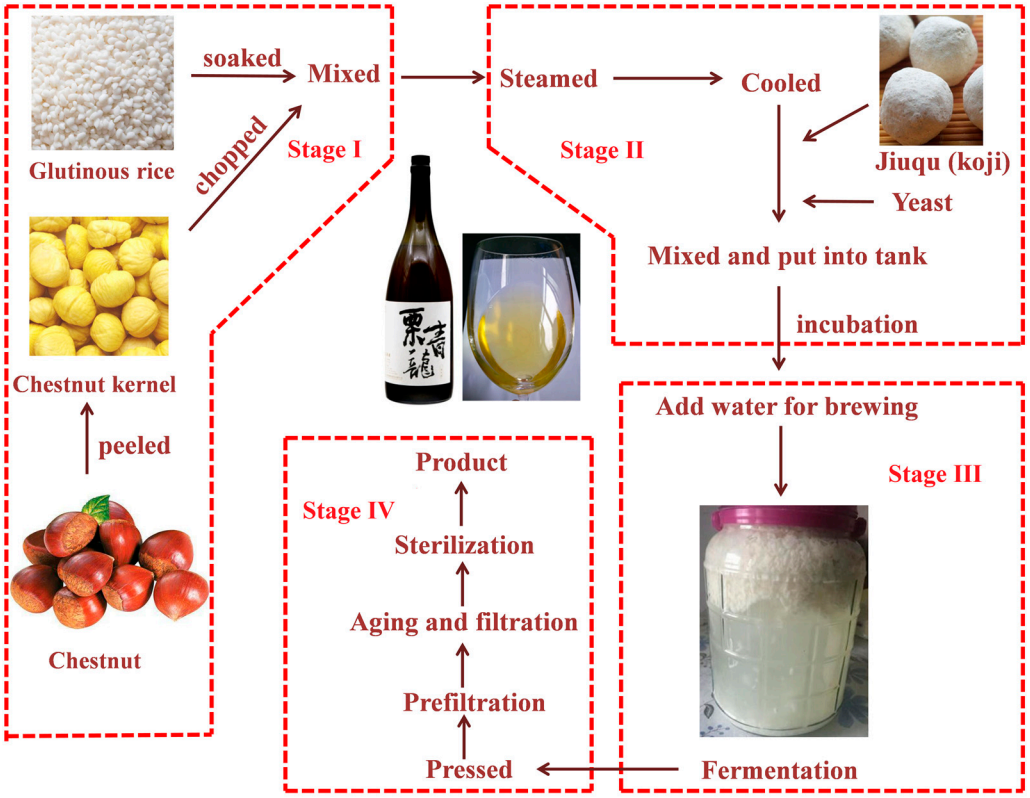

Figure 1. Schematic diagram of the BLMJ brewing process. The whole brewing process is performed in four stages. In Stage I, the glutinous rice is soaked in water and the chestnut is peeled and chopped. In Stage II, the treated chestnut and rice are mixed in a 1:3 (w/w, dry weight) ratio and steamed. After cooling to 35 °C, 4.0% (w/w) jiuqu starter culture and 0.2% (w/w) active dry yeast are added. In Stage III, water is added to the inoculated chestnut/rice mix, which is then fermented. In Stage IV, the fermented rice wine is filtered and sterilized, and the filtrate is then sealed and stored at 12 °C for 9 months. The production of traditional glutinous rice wine (TGRW) is the same as that of BLMJ, except no rice is substituted with chestnut.

Stage III, the main fermentation stage, is divided into pre-fermentation and post-fermentation stages. In pre-fermentation, the 3 kg chestnut–rice–jiuqu mixture is placed in a 20 L tank and incubated for 24 h at 30 °C. Water is then added at a 1:1.5 ratio (i.e., 1 kg initial raw material and 1.5 kg water), and the pH is adjusted to 4.2 with lactic acid. Fermentation is then performed at 25 °C for 9 d in a static state until the liquid levels stabilize. In days 3 through 9, the mixture is manually stirred every 24 h. After 9 days, entering the post-fermentation stage begins, and the tank is sealed and kept at 18 °C for 20 days. In Stage IV, the aging stage, following the main fermentation, the rice wine is filtered using a plate-and-frame filter press and sterilized at 80 °C for 10 min. The vessels used to store the BLMJ are sterilized by rinsing with boiling water and soaking for 20 min. After the first sterilization, the filtrate is sealed in boiled vessels and stored at 12 °C for 9 months. Then, the wine is bottled and sterilized a second time. In the second sterilization, sealed bottles are placed in a hot water bath (80–90 °C) for 20 min.

The process of traditional glutinous rice wine (TGRW) is similar to banli mijiu (BLMJ) fermentation, except for a 1:1 w/w substitution of chestnut kernels for rice in the initial

steps. Using this fermentation process, three separate batches (replicates) of BLMJ and TGRW were produced.

2.2. Analysis of Free Amino Acid (FAAs)

According Miao's method with slight modification [20], a 1 mL rice wine sample was added to a 125 mL round bottom flask. It was then left to evaporate for 60 min at 50 °C until the sample was dry. A loading buffer (2 mL) was added to the flask to dissolve the evaporated rice wine sample (loading buffer: 9.6 g citric acid, 8.5 g lithium citrate, 1 g phenol, 80 mL thiodiglycol, 16 mL concentrated hydrochloric acid added to 500 mL of distilled water, adjusted to a pH of 2.2, then adjusted to a volume of 1000 mL). The resulting solution was extracted with a 2.5 mL syringe, filtered with a 0.45 μm filter membrane needle filter, and stored at 4 °C until analysis. The free amino acid was determined using a Biochrom 30+ automatic amino acid analyzer (Biochrom Ltd., Cambridge, UK). All assays were performed in triplicate.

2.3. Determination of Total Phenol and Monophenol Contents

According to the method of Lingua et al. to determine the total phenol content with some modification [21], the reaction system to determine total phenol content included a 250 μL sample of rice wine, 12.5 mL distilled water, 1.25 mL Folin–Ciocalteu reagent, and 5 mL Na_2CO_3 (20%, w/v). The mixture was kept at room temperature for 60 min, after which the absorbance at 765 nm was measured. Then the total phenol content was calculated according to the standard curve, with gallic acid as the standard. The UPLC–MS/MS system was used to determine the monophenol content according to the method used by Belmiro et al. [22].

2.4. Determination of Total Flavonoids

The method used by Eberhardt et al. [23] was used to determine the total flavonoids with some modifications. A 1 mL rice wine sample and 1 mL $NaNO_3$ (5%, w/v) were mixed to react for 6 min, after which 1 mL Al $(NO_3)_3$ (10%, w/v) was added and reacted for 6 min. Then, 10 mL 1 mol/L NaOH solution and 12 mL distilled water were added. The samples were mixed and reacted at room temperature for 15 min, while the absorbance was measured at 505 nm. The flavonoid concentration was calculated according to the rutin standard curve.

2.5. Determination of the Ethyl Carbamate (EC) Content

Headspace solid-phase microextraction followed by gas chromatography–mass spectrometry (HS-SPME-GC/MS) was used to determine the EC content according to the method used by Horri and Goto [24]. An 8 mL wine sample was placed into a 20 mL vial spiked with 200 μL of EC-d5 (10 mg/L), which was used as the internal standard. The sample was capped with a PTFE/silicone septa and heated to 71 °C for 10 min. The HS-SPME analysis of the sample was performed at 73 °C for 20 min with agitation (550 rpm) using a PA fiber (Polyacrylate, 85 μm, Supelco). After the extraction step was complete, the fiber was inserted into the injection port of the GC for 10 min.

2.6. Determination of the Total Reducing Sugar (TRS), Amino acid Nitrogen (AAN), and Titratable Acidity (TA)

The total reducing sugar content of the supernatant wine was determined using the 3,5-dinitrosalicylic (DNS) acid colorimetry method. Reference analyses for TA and ANN were in accordance with the official analysis methods for Chinese rice wine (GB/T 13662-2018). The wine samples (10 mL) were briefly mixed with 50 mL of distilled water and titrated to pH 8.2 with 0.1 M NaOH, while the volume of consumed NaOH was recorded in order to determine TA content (calculated by lactic acid). A total of 10 mL formaldehyde solution (37–40%) was then added, and it was titrated to pH 9.2 with 0.1 M NaOH, while the volume of consumed NaOH was recorded to determine AAN content. A blank test was

performed using distilled water. These determinations were performed in triplicate; results are based on the average of the three.

2.7. Determination of the Antioxidant Ability

2.7.1. Determination of Reducing Power

Different volumes (50, 100, 150, 200, 250 μL) of rice wine samples were supplemented with distilled water to 1 mL and mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (*w*/*v*) solution of potassium ferricyanide. The reaction time, temperature, and determination method were all performed according to the methods used by Siddhuraju [25]. A solution of 1 g/L BHT (2,6-di-tert-butyl-4-methylphenol) and ascorbic acid was used as a positive control. All assays were performed in triplicate.

2.7.2. Radical Scavenging Activities on DPPH•

The DPPH• scavenging activity of the prepared wine samples was measured via the method used by Barros et al. [26]. The DPPH• solution (0.01%) in ethanol was prepared, and 2 mL of this solution was mixed with different volumes of rice wine (50, 100, 150, 200, 250 μL). It was then supplemented with distilled water to 4 mL. The mixture reacted for 30 min in 30 °C water. The absorbance was measured at 517 nm. BHT (0.1 g/L) and ascorbic acid were used as reference material. All assays were performed in triplicate. The scavenging activity was calculated as follows:

$$\text{Scavenging activity } (\%) = (1 - \frac{A_{sample+DPPH} - A_{sample}}{A_{DPPH}}) \times 100\% \quad (1)$$

where $A_{sample+DPPH}$ includes DPPH• and liquor samples, A_{sample} includes liquor samples but ethanol instead of DPPH•, and A_{DPPH} includes with DPPH• but no sample solution.

2.7.3. $ABTS^+$ Radical Scavenging Assay

The ability of scavenging $ABTS^+$ radicals of BLMJ and TGRW was measured according to the methods used by Ye [27], with some modifications. The $ABTS^+$ radical solution was prepared according to the methods used by Ye [27].

Different volumes of liquor samples (10–90 μL) were combined with distilled water to 0.1 mL and added to 4.9 mL $ABTS^+$ radical solution. They were then mixed vigorously. After reacting for 5 min at room temperature without light, the absorbance at 734 nm was measured. Distilled water was used as a control solution, and 1 g/L ascorbic acid and 1 g/L tocopherol were used as positive controls. Each sample was determined three times, and the $ABTS^+$ radical scavenging was calculated using the following formula:

$$\text{Scavenging rate } (\%) = (1 - \frac{A_{sample}}{A_{blank}}) \times 100\% \quad (2)$$

2.7.4. Hydroxyl Radical (•OH) Scavenging Activity

The hydroxyl radical scavenging activity of the liquor samples was determined according to the method used by Que [28], with some modifications. Each sample (1 mL) contained different amounts of rice wine (0.1–0.9 mL) and distilled water and was added to 0.75 mL 7.5 mM $FeSO_4$ solution, 2.5 mL pH 7.4 PBS solution, 1 mL 0.1% H_2O_2, and 0.75 mL 7.5 mM phenanthroline. The samples were mixed and reacted at 37 °C for 60 min, while the absorbance was measured at 536 nm. Ascorbic acid (1 g/L) was used as a positive control. Each sample was determined three times and hydroxyl radical scavenging activity was calculated as follows:

$$\text{Hydroxyl radical scavenging co-efficient } (\%) = \frac{A_{sample} - A_{damage}}{A_{blank} - A_{damage}} \times 100\% \quad (3)$$

where A_{blank} includes distilled water instead of liquor sample and H_2O_2, A_{damage} includes H_2O_2 but no liquor sample, and A_{sample} includes liquor sample and H_2O_2.

2.7.5. HS-SPME-GC/MS-O Determination of the Volatile Profile

Sample storage and preparation: The TGRW and BLMJ samples were stored at 4 °C before analysis. For both wines, 1 µL 2-octanol internal standard and 2g NaCl were added to 6 mL of each sample in a 20 mL vial and tightly capped with PTFE/silicone septum. Carboxen/polydimethylsiloxane (CAR/PDMS) CAR/PDMS SPME fibers (75 µm, Fused Silica 24 Ga, Manual Holder, 3 pk, Supelco, Bellefonte, PA, USA) were used for volatile compound extraction at 50 °C for 50 min. Compounds were desorbed for 3 min at 280 °C in splitless mode, using a 0.75 mm dedicated SPME liner.

Gas chromatography conditions for SPME-MS: an Agilent 7890 GC system was used with an HP-INNOWAX capillary column (60 m × 0.25 mm × 0.25 µm, Agilent Technologies, Foster City, CA, USA). Helium flow was 1 mL/min (isothermal), and the injector temperature was 250 °C. The initial oven temperature was 50 °C for 2 min, then increased by 2 °C/min to 115 °C for 3 min, 4 °C/min to 200 °C, and 6 °C/min to 230 °C for 10 min.

MS operating conditions: The spectrometer was run in electron impact (EI) mode (70 eV) with 1871 eV multiplier voltage. The ion source was indirectly heated by transfer line set to 280 °C. Detection was carried out in scan mode over a range of 50–550 amu. Each sample was run in 3–5 replicates for SPME-MS and 2–3 replicates for SPME-GC/MS.

Qualitative and semi-quantitative analysis: The NIST11, W8N08, and WILEY7N standard spectral libraries were searched by Agilent Technologies MSD Chemstation D.03.00.552 chemical workstation, and the standard spectra of relevant compounds were confirmed with spectral data from published studies. The retention indices (RI) values of each compound were calculated using the direct paraffin method and compared with values in the literature. Then, the aroma characteristics of each compound in TGRW and BLMJ were identified using gas chromatography olfactometry (GC-O).

The GC-O with Gerstel ODP3 sniffer (Gerstel Trading Co., Ltd., Shanghai, China) study included five panelists with more than five years of sensory analytical experience in alcoholic beverages. All panelists were familiar with the GC-O technique and had been trained in GC-O analysis twice per week for more than six months. Panelists responded to and recorded the retention time and descriptor for each aromatic compound using a six-point scale ranging from 0 to 0.5 to rank aroma intensity (0 = none, 0.1 = slight, 0.3 = moderate, 0.4 = strong, and 0.5 = extreme). Each sample was smelled twice by each panelist. Aroma intensity values obtained from the two tests were averaged for each of the five panelists, resulting in a total of 10 analyses per compound [29].

3. Results and Discussion

3.1. Metabolite and Nutrient Content of BLMJ

3.1.1. Chemical Indexes of BLMJ

The major constituents of BLMJ were examined for comparison with that of TGRW to determine whether the addition of chestnut changed any of the fundamental qualities of traditional rice wine. The assay showed that the ethanol content of BLMJ was 15.11% (*v*/*v*), while titratable acidity, total reducing sugars, and amino acid nitrogen of BLMJ were 2.72 g/L, 1.34 g/L, and 0.21 g/L, respectively, none of which were significantly different than that of TGRW (Table 1), thus suggesting that the major components of TGRW remained unaffected by substitution of chestnut for rice as a feedstock in brewing.

Table 1. The main index of BLMJ and TGRW.

Names	Ethanol Content (%, *v*/*v*)	Titratable Acidity (g/L)	Reducing Sugar (g/L)	Amino Acid Nitrogen (g/L)
BLMJ	15.11 ± 0.21 [a]	2.72 ± 0.16 [a]	1.34 ± 0.16 [a]	0.21 ± 0.08 [a]
TGRW	14.25 ± 0.18 [a]	3.09 ± 0.20 [a]	2.34 ± 0.10 [a]	0.28 ± 0.01 [a]

[a] in the same column indicates means without significant difference by Duncan's post hoc tests at $p = 0.05$.

3.1.2. Phenol and Total Flavonoid Profile

Chestnuts were found to be rich in polyphenols including ellagic acid, gallic acid, and others [6–9]. To compare phenolic profiles between BLMJ and TGRW, total phenolic contents of each wine were determined, with gallic acid serving as an internal standard to generate a standard curve for phenol quantitation (Table 2). The result showed that total phenol content in BLMJ was 187.1 μg/mL, which was significantly higher than that in glutinous rice wine (113.2 μg/mL) ($p < 0.05$). To determine the species and contents of each individual monophenol, UPLC–MS/MS was used, which successfully identified seven phenolic species in BLMJ compared to four species found in TGRW (Figure 2). The caffeic acid, gallic acid, and (+) catechin were only present in BLMJ. Among the other four common monophenolic species, the contents of trans-p-coumaric acid and trans-4-hydroxy-3-methoxycinnamic acid were significantly higher in BLMJ than that in TGRW.

Table 2. Linear regression of gallic acid and rutin.

Name	Regression Equation	R^2
Gallic acid	y = 0.0012x + 0.0598	0.9977
Rutin	y = 0.5036x − 0.0069	0.9985

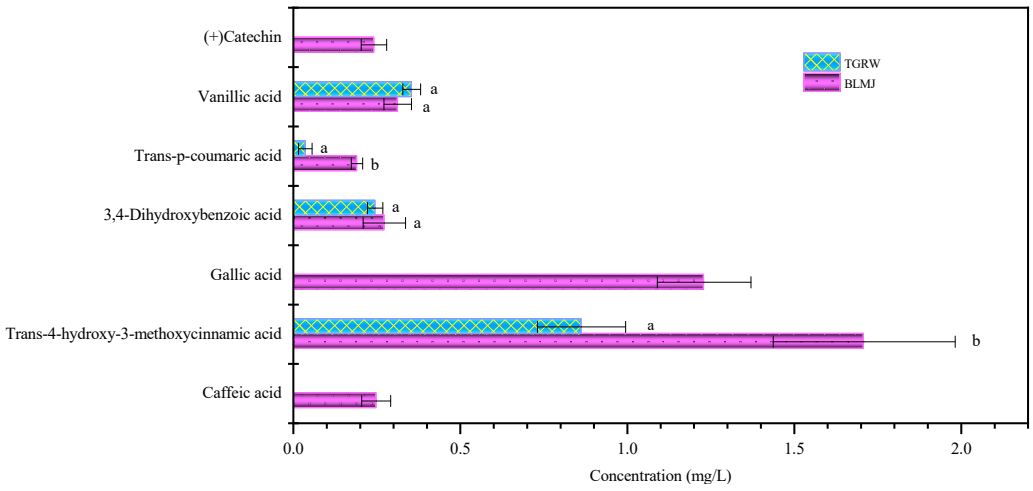

Figure 2. The contents of monophenolic species in BLMJ and TGRW. Ultra-high-pressure liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) analysis was used to quantify phenolics in samples of BLMJ and TGRW. Caffeic acid, gallic acid, and (+) catechin were only present in BLMJ. Data show the means ± SD of three replicates. Significance was determined by Duncan's post hoc test, and a and b in each group indicate means with significant difference at $p < 0.05$.

Apart from phenols, the concentration of flavonoids was then examined. Using the chromogenic method, the flavonoid content of each wine was determined, with rutin used to generate a standard curve (Table 2). This assay showed that the total flavonoid content in BLMJ was 106.5 μg/mL, significantly higher than that of TGRW (80 μg/mL) ($p < 0.05$). Together with results of phenol quantification, these data showed that partial replacement of glutinous rice with chestnut in the brewing process can significantly increase the contents of these compounds.

3.1.3. Free Amino Acid (FAA) Content of Banli Mijiu (BLMJ)

The amino acid profile contributes to both flavor and aroma of banli mijiu (BLMJ) as well as its nutrient content. Content analysis of individual amino acids revealed that BLMJ contains the same suite of 18 peptide amino acids and 7 non-peptide amino acids found in traditional glutinous rice wine (TGRW) (Table 3), but at significantly lower total concentrations in BLMJ than that in TGRW (4354.924 vs. 6588.456 mg/L; $p < 0.05$). Apart from tryptophane, tyrosine, histidine, and proline, all other amino acids were found in significantly higher concentrations in TGRW compared to BLMJ ($p < 0.05$). Among non-peptide amino acids, no significant differences between wines in β-alanine, homocysteine (Hcy), and γ-aminobutyric acid (GABA) contents were found, whereas the hydroxylysine, citrulline, and ornithine contents were significantly higher in TGRW than that in BLMJ ($p < 0.05$) (Table 3). In addition, the ratio of total umami and sweet FAAs to bitter FAAs were 1.022 in BLMJ, which was 114.3% higher than that of TGRW. These differences between the two rice wines were likely due to the lower protein content of chestnuts, which was substituted 1:1 w/w for glutinous rice in ≈25% of the fermentation input.

Table 3. The protein amino acid and non-peptide amino acids species and contents in BLMJ and TGRW.

Amino Acid Types	Names	BLMJ (mg/L)	TGRW (mg/L)
Protein amino acid	Asp	155.407 ± 6.642 [a]	209.202 ± 8.119 [b]
	Thr	89.622 ± 3.848 [a]	130.651 ± 4.003 [b]
	Ser	143.433 ± 9.809 [a]	194.494 ± 2.019 [b]
	Asn	159.797 ± 7.740 [a]	206.526 ± 10.060 [b]
	Glu	416.867 ± 12.192 [a]	519.314 ± 29.173 [b]
	Gly	203.410 ± 2.359 [a]	245.834 ± 11.454 [b]
	Ala	347.460 ± 16.590 [a]	465.710 ± 10.303 [b]
	Val	253.379 ± 5.264 [a]	351.462 ± 21.431 [b]
	Met	85.665 ± 7.718 [a]	123.781 ± 16.923 [b]
	Ile	122.485 ± 9.578 [a]	177.597 ± 15.362 [b]
	Leu	284.398 ± 18.195 [a]	420.525 ± 29.897 [b]
	Tyr	319.582 ± 1.801 [a]	323.910 ± 13.754 [a]
	Phe	259.755 ± 13.370 [a]	365.215 ± 20.674 [b]
	His	97.248 ± 6.852 [a]	126.009 ± 16.845 [a]
	Lys	189.366 ± 5.006 [a]	263.570 ± 14.796 [b]
	Try	39.288 ± 4.962 [a]	57.066 ± 1.209 a
	Arg	90.637 ± 9.260 [a]	346.351 ± 2.012 [b]
	pro	238.691 ± 15.508 [a]	284.400 ± 23.342 [a]
	Umami FAAs	572.274 ± 6.801 [a]	728.516 ± 37.280 [b]
	Bitter FAAs	1645.098 ± 5.958 [a]	2431.705 ± 98.043 [b]
	Sweet FAAs	1108.280 ± 35.182 [a]	1444.871 ± 24.341 [b]
Non-peptide amino acids	Citrulline	31.402 ± 2.565 [a]	75.304 ± 2.978 [b]
	Cystine	35.113 ± 3.528 [a]	53.147 ± 2.147 [b]
	β-Alanine	56.368 ± 0.646 [a]	51.632 ± 5.878 [a]
	Hydroxylysine	404.169 ± 15.731 [a]	937.774 ± 26.285 [b]
	Omithine	147.892 ± 11.110 [a]	482.584 ± 7.533 [b]
	γ-Aminobutyric acid	162.108 ± 14.328 [a]	157.255 ± 15.722 [a]
	Homocysteine	21.512 ± 0.346 [a]	20.267 ± 1.959 [a]
	SUM FAAs	4354.924 ± 72.536 [a]	6588.456 ± 166.261 [b]

Note: All values were means of triplicate determinations ± SD. Means within different letters were significantly ($p < 0.05$) different on the same line among the contents of a certain amino acid. Umami FAAs (Glu and Asp), sweet FAAs (Thr, Ser, Gly, Ala, Pro, and Met), and bitter FAAs (Tyr, Ile, Leu, Val, Phe, Lys, His, Try, and Arg). FAAs: free amino acids.

For brewing rice wine, the selection of raw materials with high starch but low fat content is essential to produce a high-quality wine with a desirable sensory profile [30] (i.e., a complex aroma and mild flavor) and ethanol content. In addition to starch, Chinese rice wine needs an appropriate protein content in the raw ingredients to provide sufficient

nitrogen for the growth of yeast during fermentation, but not at levels which result in amino acid accumulation from degraded proteins [30]. Other studies have shown that fusel alcohol contents in the finished rice wine are positively correlated with protein content in the raw materials [31]. The fusel alcohols greatly contribute to the flavor of rice wine and promote the correct aroma and mouthfeel. However, excess fusel alcohols can potentially result in acute toxicity and exert neurotoxic effects [32]. While the reduced amino acid content may result in a more subtle flavor and mild aromatic profile for BLMJ, the comparatively low amino acid content may also benefit the production process, since excess amino acids, especially arginine, can lead to the toxic accumulation of ethyl carbamate during traditional rice wine fermentation. In light of these differences in amino acid contents, we therefore next determined the EC contents in BLMJ and TGRW.

3.1.4. Ethyl Carbamate Content

The accumulation of the fermentation by-product ethyl carbamate (EC) has been reported in numerous food and alcohol products, including cheese, bread, yogurt, wine, whiskey, soy sauce, and others [33]. However, EC has been described as a potential health risk and likely carcinogen among some frequent consumers of alcohol [33,34]. In other work, EC was revealed to serve as a significant risk factor for lung cancer development, as well as bronchioalveolar adenomas and non-small cell lung carcinoma [35]. To compare the concentrations of EC between BLMJ and TGRW, the EC was quantified using HS-SMPE-GC/MS techniques. The results of this assay showed that the EC content in BLMJ was 142.13 mg/L, significantly lower than that in traditional rice wine (209.56 mg/L; $p < 0.05$). This finding was unsurprising, given the significantly higher arginine content in TGRW compared to that in BLMJ, which might result from the cooking process, as Li and colleagues previously verified that cooking Chinese chestnut (including boiling, roasting, or frying) could significantly decrease its arginine contents [36]. Arginine has been shown to lead to the accumulation of decomposition products such as ornithine and urea in yeast cells [37]. Urea, in particular, serves as a precursor of ethyl carbamate, and TGRW had significantly higher levels of accumulated ethyl carbamate, reflecting the higher levels of arginine in the starting materials.

3.2. Antioxidant Activity of Banli Mijiu (BLMJ) Chestnut Rice Wine

3.2.1. Reducing Power

Chestnut and their by-products can be used as food additives in food products to delay oxidation processes and improve quality characteristics [4,6–9]. Reducing power can serve as an indicator of potential antioxidant activity, so the reducing power of BLMJ was quantified for comparison with TGRW and Vc or BHT reducing agent controls through measurement of antioxidant electron donating activity using the Prussian blue method (Fe^{3+} to Fe^{2+}) for colorimetric detection of substrate reduction. Both BLMJ and TGRW exhibited the ability to reduce the test substrate, which gradually increased in a dose-dependent manner with the addition of increasing volumes of either wine (Figure 3a). Although significantly lower than that of the vitamin C and BHT control antioxidants, BLMJ exhibited significantly higher reducing power than that of TGRW, strongly suggesting that the addition of chestnut as a feedstock for rice wine improved its capacity for antioxidant activity.

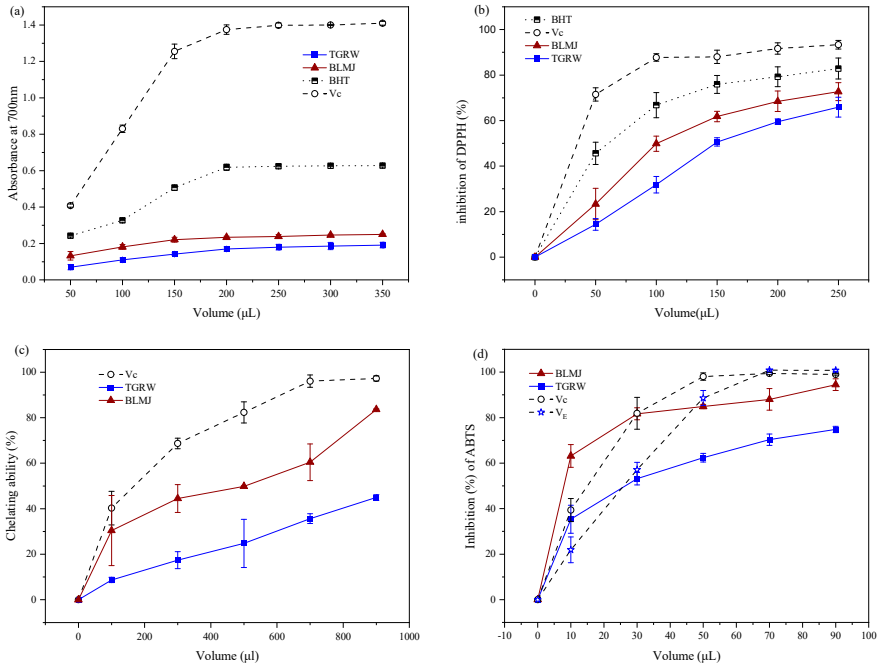

Figure 3. (**a**) Reducing power of banli mijiu (BLMJ), traditional glutinous rice wine (TGRW), and synthetic antioxidants butylated hydroxytoluene (BHT) and ascorbic acid (vitamin C; Vc). Each sample was assayed in triplicate at each volume. (**b**) DPPH• free radical scavenging activity of BLMJ and TGRW. BHT and Vc were used as controls. (**c**) Absorbance of hydroxyl radicals by BLMJ, TGRW, and Vc. All data are means ± SD for three measurements. (**d**) Inhibitory effects of BLMJ, TGRW, ascorbic acid (vitamin C; Vc), and tocopherol (vitamin E; Ve) on $ABTS^+$ radical.

3.2.2. DPPH• Free Radical Scavenging Rate

2,2-Diphenyl-1-picrylhydrazyl (DPPH•) is a stable free radical, and the capacity to scavenge DPPH• is a standard technique for evaluating the inhibition of lipid oxidation by food products [38]. This experiment thus reflects the ability of BLMJ scavenge free radicals on the basis of the accumulation levels of diphenylpicryl hydrazine, the reduced DPPH• reaction product. The results showed that both wines and both controls were able to scavenge DPPH• radicals in a dose-dependent manner. While vitamin C had the strongest ability, followed by BHT, BLMJ had significantly higher DPPH• scavenging activity than that of TGRW (Figure 3b). As volumes of the two added wines reached 200 μL, the scavenging of DPPH• radicals plateaued for all of the four samples. At this volume, BLMJ and TGRW scavenged 38.67% and 23.36% of the DPPH•, respectively. The higher capacity for scavenging DPPH• was possibly due to carryover of vitamins, phenols, or other metabolites that can neutralize free radicals from the chestnut feedstock that are also retained by the rice wine.

3.2.3. Capacity for Absorbance of Hydroxyl Radical

Hydroxyl radicals (•OH) are reactive oxygen species produced by light, radiation, or metabolic processes that exhibit strong electron-trapping activity and cause oxidative damage to tissues and cells. These reactive oxygen species have been associated with the progression of cancer, aging, and lung diseases [39,40]. The capacity to scavenge hydroxyl radicals is considered a reliable indicator of the antioxidant activity of foods. The Fenton system was used to determine the •OH scavenging ability of BLMJ and TGRW.

The results of this assay revealed that both rice wines were able to scavenge hydroxyl radicals, although to a lesser extent than vitamin C, and that the capacity for •OH clearance was positively correlated with antioxidant dose, up to 900 μL in 5 mL reaction volume (Figure 3c). As with other free radicals, BLMJ exhibited significantly greater scavenging activity than TGRW ($p < 0.05$), and at 0.9 mL dosage, BLMJ cleared 83.48% of the available

•OH, whereas TGRW cleared only 45.65%. Collectively, these results indicate that the use of chestnut as a supplement to the fermentation feedstock in the brewing process increases the antioxidant properties of TGRW by enhancing the capacity for scavenging different types of free radicals and reactive oxygen species. Chestnut is rich in a variety of vitamins and phenols such as gallic acid and ellagic acid [1,3]. Although a significant decrease in the content of vitamin C in chestnut occurs during the steaming process [26], ascorbic acid converts to dehydroascorbic acid, thereby providing relatively stronger antioxidant properties, which was supported by observations of BLMJ scavenging of DPPH•, •OH, and $ABTS^+$ radicals.

3.2.4. $ABTS^+$ Free Radical Scavenging Ability

The interaction between antioxidant substances and $ABTS^+$ reveals their ability to scavenge hydroxyl radical moieties that are specifically attached to aromatic rings [41]. The results showed that BLMJ, TGRW, and the controls (vitamin E and vitamin C) could scavenge $ATBS^+$ in a manner positively correlated with dose. At 10 µL in 5 mL reactions, the scavenging ability of BLMJ was significantly higher than that of TGRW, vitamin C, and vitamin E ($p < 0.05$), although the clearance rate at 70 µL and 90 µL of BLMJ was significantly lower than the antioxidant controls, vitamin C and vitamin E ($p < 0.05$) (Figure 3d). These results indicated that the free radical scavenging effects of BLMJ were more obvious at low doses. The $ABTS^+$ removal rate of BLMJ remained significantly higher than that of TGRW ($p < 0.05$), indicating that the chestnut component significantly increased the free radical scavenging ability of rice wine. Moreover, the clearance rate of 90 µL BLMJ was 94.50%.

3.3. Flavor Compounds and Odor Descriptors

The addition of chestnut substantially influenced the range and abundance of flavor-related compounds compared to that in TGRW. In total, 98 distinct flavor components were identified in the BLMJ, compared to 77 distinct flavor components in TGRW (all flavor compounds identified in both BLMJ and TGRW are listed in Table S1), with the largest differences found in the number of alcohols, olefins, and benzenes. Among these flavor components, there were 38 compounds detected by the GC-O sniffer instrument in BLMJ and 22 in TGRW (Table 4). Among the 38 compounds, 6 alcohols, 7 olefins, 7 esters, 5 carboxylic acids, 4 ketones, 4 aldehydes, 3 phenolic compounds, and 2 benzenes were sniffed in BLMJ (Table 4).

Table 4. Flavor materials identified using gas chromatography olfactometry (GC-O) in BLMJ and TGRW.

Names	Retention Time (RT)	RI	BLMJ		TGRW		Odor Description
			Content (µg/L)	Odor Intensity	Content (µg/L)	Odor Intensity	
Alcohols				6		7	
Ethanol	8.9577	972.4373	206.984	0.5	260.293	0.4	alcoholic
2-Methyl-1-propanol	14.8053	1120.2487	4.461	0.4	0.583	0.2	solvent
3-Methyl-1-butanol	21.0876	1233.3929	53.079	0.5	51.332	0.5	whiskey
β-Phenylethyl alcohol	56.2091	1930.7626	17.297	0.2	26.647	0.4	floral, rose
3-(Methylthio)-1-propanol	49.5738	1729.0585	0.137	0.1	0.117	0.1	sweet, onion
1-Hexanol	29.3376	1365.0278	0.314	0.4	-		fusel, sweet alcoholic
3,7-Dimethyl-1,6-octadien-3-ol	41.8238	1561.6868	-		0.499	0.2	rose, blueberry
α-Terpineol	48.8775	1709.5367	-		0.539	0.2	clove, citrus

Table 4. *Cont.*

Names	Retention Time (RT)	RI	BLMJ		TGRW		Odor Description
			Content (μg/L)	Odor Intensity	Content (μg/L)	Odor Intensity	
Olefins				7		3	
(1R)-(+)-α-Pinene	11.7752	1053.4724	17.014	0.1	0.821		minty
(+)-4-Carene	18.8289	1193.5565	1.239	0.1	0.001		citrus, pineapple
D-Limonene	20.4698	1222.6191	441.934	0.3	21.071	0.1	citrus
γ-Terpinene	22.5454	1257.6133	0.051	0.1	0.771	0.1	lemon, citrus
Naphthalene	50.8224	1763.3878	0.105	0.1	0.092	0.1	fuel
2-Carene	18.5021	1188.2175	3.644	0.1	-		citrus, pineapple
α-Terpinolene	25.1472	1297.1855	8.105	0.1	-		lemon, citrus
Esters				7		6	
Ethyl acetate	7.6731	923.1871	1.498	0.1	2.087	0.2	fruity, sweet
3-Methyl-1-butanol acetate	15.678	1137.7131	0.277	0.1	0.839	0.2	sweet, banana
Ethyl hexanoate	21.9244	1247.493	0.519	0.3	1.354	0.3	sweet pineapple
Ethyl decanoate	46.9491	1664.9882	0.443	0.3	0.198	0.1	fruity, brandy
2-Phenylethyl acetate	53.4536	1840.7278	1.912	0.4	1.217	0.2	rose, honey, fruity
Ethyl hexadecanoate	64.9821	2300.7646	0.623	0.5	0.589	0.3	fruity, cream
Ethyl tetradecanoate	60.491	2090.4304	0.021	0.4	-		floral, violet
Carboxylic acids				5		1	
Benzoic acid	68.6528	2461.6125	0.239	0.2	0.502	0.2	phenolic
Acetic acid	35.7507	1462.8206	0.396	0.1	-		vinegar
Formic acid	39.8725	1527.6034	0.007	0.1	-		vinegar
2-Methyl-propanoic acid	42.8665	1579.2532	0.233	0.1	-		yogurt, milk, cream
Octanoic acid	59.9975	2071.302	0.305	0.2	-		vegetable, cheese
Ketones				4		1	
2-Methyl-5-(1-methylethenyl)-trans-cyclohexanone	45.2853	1627.1005	0.102	0.1	-		pepper
D-Carvone	50.5315	1755.4659	0.297	0.1	-		bread, coriander
1-(4-Methylphenyl)-ethanone	52.0122	1795.3246	2.166	0.1	-		cherry, mimosa, acacia
1-(3-Methylphenyl)-ethanone	52.2475	1801.9149	0.460	0.1	-		alfalfa, honey,
1-(4-Methylphenyl)-ethanone	52.1658	1799.3943	-		1.251	0.2	acacia, coumarin
Aldehydes				4		1	
Benzaldehyde	40.7419	1542.9907	0.121	0.2	0.049	0.1	almond
Furfural	36.764	1477.5765	0.425	0.1	-		almond, toasted bread
3-Methyl-benzaldehyde	47.1157	1668.7078	0.155	0.1	-		cherry, bitter almond
2-Methyl-3-phenyl-2-propenal	57.0753	1961.2853	0.490	0.1	-		sweet, cinnamon

Table 4. *Cont.*

Names	Retention Time (RT)	RI	BLMJ		TGRW		Odor Description
			Content (μg/L)	Odor Intensity	Content (μg/L)	Odor Intensity	
Phenolic compounds				3		1	
Phenol	58.7979	2024.1401	0.189	0.1	0.524	0.2	phenolic
Carvacrol	62.9654	2201.4761	0.262	0.1	-		spicy, woody
Thymol	63.6257	2234.3396	0.122	0.1	-		thyme
Benzenes				2		2	
1-Methyl-4-(1-methylethenyl)-benzene	34.9793	1451.3041	12.772	0.2	4.119	0.1	clove, coffee, nutty
o-Cymene	24.2353	1283.8022	102.491	0.2	3.260		citrus, woody
p-Cymene	24.2451	1283.9487	-		3.438	0.1	citrus, woody

Different from TGRW, quantification of olefins showed the largest difference between BLMJ and TGRW in this study. As seen in Table S1 and Table 4, there were 27 species of olefins identified by HS-SPME-GC/MS in BLMJ, with D-limonene, α-terpinene, β-pinene, (1R)-(+)-α-pinene, and (+)-4-carene showing the highest concentrations. Among them, seven species of olefins were detected by sniffer instrument, and the odor intensity of D-Limonene was the highest (Table 4). By contrast, 17 olefins were identified by mass spectrometry in TGRW, 3 of which could be detected by GC-O sniffer (Table 4). Other studies have shown that free terpenes, in the form of hydrocarbons, alcohols, phenols, ketones, aldehydes, and esters, are typically the main aromatic compounds in fruit wines [42]. Monoterpenes can impart floral and fruity aromas to wine and are derived from both the raw material (grapes) and the fermenters (non-*Saccharomyces cerevisiae* yeasts and some filamentous fungi). Since BLMJ and TGRW are both made using the same process and the same type and amount of koji, the differences in certain terpenes between the two wines can be attributed to the addition of chestnut as a raw material.

Quantification of alcohols in BLMJ and TGRW showed that, in total, HS-SPME-GC/MS identified 14 alcohols in BLMJ and 19 in TGRW, among which ethanol and 3-methyl-1-butanol were the main alcohols in both beverages (Table S1). In addition, 2-methyl-1-propanol, 3-methyl-1-butanol, and 1-hexanol were the main alcohols detected by GC-O sniffer in BLMJ (the odor intensity values were 0.4, 0.5, and 0.4, respectively), while β-phenylethyl alcohol and 3,7-dimethyl-1,6-octadien-3-ol were the main alcohols sniffed in TGRW (Table 4). These alcohols largely contribute to sweet, floral, and rose aromas, according to Chen and coworkers [43]. The contents of different alcohols are influenced by the particular strains of yeast and sugars used for fermentation, as well as the catabolism of amino acids in raw material, and thus differences in their contents between TGRW and BLMJ in this study could be explained by the use of chestnut as a raw material [31,44]

Ester components were also quantified by mass spectrometry and sniffer instrument in this study. A total of 12 esters were identified in BLMJ (Table S1). On the basis of their odor intensity, ethyl hexadecanoate, 2-phenylethyl acetate, ethyl tetradecanoate, ethyl hexanoate, and ethyl decanoate were potentially important esters for the aromatic profile of BLMJ (Table 4). The accumulation of certain esters is not only related to specific yeast strains, but also to the brewing environment, fermentation conditions, and raw materials [29,44], which was related to the addition of chestnut in BLMJ, as with other compounds identified above.

Quantification of volatile carboxylic acids in BLMJ revealed benzoic acid, octanoic acid, acetic acid, formic acid, and 2-methyl-propanoic acid as the main carboxylic acids that affected the aromatic profile detected by GC-O sniffer, and the odor intensity values were above 0.1 (Table 4). In addition, carbonyl compounds including ketones and aldehydes were detected in both wines. There were 10 species of ketones and six aldehydes in BLMJ compared with five ketones and three main aldehydes in TGRW (Table S1). Among them, the most potentially important aldehyde was benzaldehyde (Table 4), which imparted

a bitter almond aroma. Benzaldehyde can be formed by the oxidation of the benzyl alcohol or by the activity of microorganisms on aromatic amino acids or phenyl acetic acid and p-hydroxybenzoic acid substrates [45]. In addition, compared with TGRW, furfural, 3-methyl-benzaldehyde, and 2-methyl-3-phenyl-2-propenal were only sniffed in BLMJ (Table 4). Benzenes are another important class of compound in BLMJ. A total of 10 benzene compounds were detected, including o-cymene and p-cymene, which contribute "ginger" and citrus attributes (Table S1 and Table 4). Other compounds such as phenols, furans, and pyridines were also detected in both rice wines, but at relatively trace levels.

4. Conclusions

In this work, the flavor-related, nutritional, and antioxidant effects of adding chestnut to glutinous rice wine were investigated through side-by-side comparison of chestnut-infused rice wine (BLMJ) with rice wine (TGRW) produced under the same conditions and ingredients except chestnut. The data showed that substitution of up to 25% of glutinous rice with chestnut (1:1 w:w) in wine fermentation had positive effects on the diversity of flavor compounds, without impacting ethanol content or other characteristics (Table 1). The levels of free amino acids were higher in TGRW than BLMJ (Table 3), which may have contributed to the higher ethyl carbamate content in the traditional wine. Moreover, BLMJ contained a wider suite of phenols and flavor components (Figure 2, Table 4), but similar alcohol, acid, and reducing sugar profiles to that of TGRW (Table 1). Gas chromatography olfactometry with mass spectrometry showed that BLMJ had a distinct flavor compound profile from that of TGRW (Table 4). The antioxidant activity was also evaluated through reducing power, metal chelation, and free radical scavenging (Figure 3). BLMJ showed greater reducing power towards $ABTS^{+}$ substrate, higher metal chelation activity, greater $DPPH^{\bullet}$ free radical scavenging activity, and higher $^{\bullet}OH$ scavenging than that of TGRW, suggesting that BLMJ retained the antioxidant properties of its raw ingredients, which were absent in TGRW produced through the same process. These findings thus highlight that the use of chestnut in fermentation increases the antioxidant properties of banli mijiu wine over that of traditional glutinous rice wine, while also increasing the number of flavor- and aroma-related compounds, potentially enhancing its sensory profile.

5. Patents

The BLMJ has been patented in China, and the patent number is ZL 201510799335.9.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8060266/s1, Table S1: Flavor materials in BLMJ and TGRW identified by headspace solid phase microextraction-gas chromatography mass spectrometry (HS-SPME/GC-MS).

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