Effects of microbial fermentation on enzyme activity and volatile properties of Massa Medicata Fermentata

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ABSTRACT
Aim: Massa Medicata Fementata (MMF) is a crude drug used in East Asia to treat anorexia and dyspepsia. It is prepared from wheat and several herbs through microbial fermentation using Aspergillus sp. and Rhizopus sp. There is great difference in the quality of commercial MMF, and the microbes of MMF are suggested to affect its quality. We investigated the effects of microbial fermentation on the quality of MMF.
Methods: Raw materials of MMF were mixed according to the ratio listed in the National Standard for Chinese Patent Drugs, and MMF was prepared using pure cultures of Aspergillus oryzae or Rhizopus oryzae. Digestive enzyme activities (α-amylase, protease, and lipase) and volatile compounds were measured using an analytical kit and GC–MS, respectively.
Results: Enzyme activity increased in MMF. MMF prepared with A. oryzae (MMF-A) showed higher α-amylase and lipase activities than that prepared with R. oryzae (MMF-R). Protease activity was marginally higher in MMF-R than in MMF-A. GC–MS analysis revealed that terpenoids decreased with fermentation; however, 2,3-butanediol, acetoin, and guaiacol were detected in MMF only. C8 compounds such as 1-octen-3-ol were higher in MMF-A than MMF-R; however, aromatic compounds such as 4-vinylguaiacol and pyrazines were higher in MMF-R than MMF-A.
Conclusion: Microbial fermentation contributes to increased enzyme activity and changes in MMF volatiles. These properties of MMF were considerably affected by the microbes used, and it is proposed in this study that it is important to have microbial control in the production of commercial MMF.

KEY WORDS: Aspergillus oryzae, GC–MS, Massa Medicata Fementata, Rhizopus oryzae, shinkiku

INTRODUCTION
Massa Medicata Fementata (MMF) is a traditional crude drug used in China (shenqu), Korea (singug), and Japan (shinkiku). MMF is prepared through microbial fermentation of wheat, apricot kernel, red beans, polygonum, sweet wormwood, and cocklebur [1]. MMF is manufactured in China and Korea; it is also used in Japanese Kampo medicine as a component of ‘hangyakujutsuitemmato’, which is used to treat dizziness and nausea [2].

MMF has been traditionally used for the treatment of anorexia and dyspepsia, and it has recently been reported to strengthen the digestive system in vivo [3,4]. MMF plays a role in regulating intestinal microbes, such as Bacteroides and Verruomicrobia, in mice [3]. Dietary supplementation with MMF was reported to enhance appetite and reduce the relative abundance of pathogens in the intestinal contents and feces of piglets [4]. Some bioactive compounds in MMF might affect the gastrointestinal tract.

Several studies have revealed that MMF has digestive enzyme activities such as those of α-amylase, protease, and lipase [5,6]. As these enzymes support the endogenous digestive enzymes in saliva, gastric juice, and pancreatic juice [7], they are thought to contribute to the medicinal effects of MMF. Alternatively, MMF has a characteristic flavor which also affects gastrointestinal function [8–10]. MMF contains various volatiles such as terpenoids, 1-octen-3-ol, 4-vinylguaiacol, and pyrazines [5]. Terpenoids are reported to have gastroprotective effects [8], and 4-vinylguaiacol and...
Pyrazines have been reported to have anti-inflammatory effects and stimulate gastrointestinal motility [9,10]. These volatiles also seemed to be another key constituent of the medicinal characteristics of MMF.

The demand for MMF has increased, along with an increase in the consumption of *hangebyakujutsutemmato*. However, the quality of commercial MMF varies greatly, and this is a serious problem for the standardization of MMF. Our previous study showed that enzyme activity, volatile composition, and microbes of MMF differ considerably with area of manufacture [5]. Although *Aspergillus* sp. and *Rhizopus* sp. have been detected in various commercial MMF [11], the ratio in which they are present was different among manufacturers [5].

*Aspergillus* sp. and *Rhizopus* sp. have been reported to secrete various enzymes and are traditionally used in the brewing industry [12]. Given that enzyme activities increase during MMF production [6], the enzymes in MMF appeared to be produced by *Aspergillus* sp. and *Rhizopus* sp. In addition, volatiles such as 4-vinylguaiacol and 1-octen-3-ol are also produced by filamentous fungi, and fungal β-glucosidase hydrolyzes β-glycosides to liberate various terpenoids [13].

As enzyme activity and volatiles partially correspond to microbial differences in commercial MMF, the differences in filamentous fungi may have a critical impact on the medicinal properties of MMF. The instability of MMF quality is a serious problem in applications where pharmaceutical grade is required. In such cases it is essential to identify the factors affecting the quality of MMF. However, to our knowledge no studies have been made yet of the relationships between the microbe and quality of MMF. Clarifying the microbiological factors involved in the quality of MMF can be useful for the quality control of its manufacturing process. Here, we investigated the effects of microbial differences on MMF enzyme activity and volatiles through MMF production using pure cultures of *Aspergillus oryzae* and *Rhizopus oryzae*.

**MATERIALS AND METHODS**

**Chemicals, strains, and plant materials**

The chemicals used for analysis were obtained from Sigma-Aldrich Japan G.K. (Tokyo, Japan), Nacalai Tesque Inc. (Kyoto, Japan), and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). *Aspergillus oryzae* (Super Ichi Murasaki) was obtained from Bioc Ltd. (Aichi, Japan), and *Rhizopus oryzae* strain NBRC 4706 was obtained from the National Institute of Technology and Evaluation (Osaka, Japan). Powdered wheat flour and bran (*Triticum aestivum* L.), red beans (*Vigna angularis* (Willd.) Ohwi et H.Ohashi var. *angularis*), and apricot kernel (the seed of *Prunus armeniaca* L.) were purchased from a local market in Japan. Cocklebur (the fruit of *Xanthium strumarium* L. subsp. *sibiricum* (Patrin ex Widder) Greuter) was purchased from China through a distributor in Japan. Sweet wormwood (aerial parts of *Artemisia annua* L.) was obtained from a local Chinese market in Chongqing. Polygonum (the aerial parts of *Persicaria hydropiper* (L.) Delarbre) was collected from Yamashina Botanical Research Institute (Kyoto, Japan). Sweet wormwood and polygonum were ground using a rotor mill (Pulverisette 14, Fritsch GmbH, Idar-Oberstein, Germany) into a powder (particle diameter >6 mm) and were used to make MMF.

**MMF production**

MMF was prepared according to the National Standard for Chinese Patent Drugs with minor modifications (Fig. 1) [1]. Wheat flour (25 g), wheat bran (50 g), polygonum (5 g), cocklebur (5 g), sweet wormwood (5 g), apricot kernel (1 g), and red beans (1 g) were mixed. Thereafter, conidia of *A. oryzae* or *R. oryzae* (9.2 × 10⁷ conidia) were inoculated into the mixture with 52 mL sterile water. The inoculated material was placed in a glass petri dish covered with a paper towel and incubated at 35°C with a relative humidity of 98% in an environmental chamber (KCL-2000A, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Following 48 h of incubation, the mixture was dried in an oven (ON-600, AS ONE.

![Figure 1](link) | The production procedure of *Massa Medica Fermentata* in this study.
corporation, Osaka, Japan) at 55°C for 15 h to produce MMF. MMF prepared with A. oryzae and R. oryzae was named MMF-A and MMF-R, respectively. The production process was repeated three times, and analytical data were obtained from three separate experiments.

**Monitoring filamentous fungi growth**

Total DNA was extracted from the incubating materials at 0, 24, and 48 h. DNA extraction was performed using a GenCheck® DNA Extraction Kit Type S/F (FASMAC Co., Ltd., Kanagawa, Japan), according to the manufacturer’s guidelines. Each 100-mg sample was disrupted at 6.0 m/s for 45 s by bead beating using a FastPrep 120 Cell Disrupter System (Thermo Savant; Carlsbad, CA, USA) and subjected to DNA extraction. Real-time polymerase chain reaction (PCR) was performed using a Thermal Cycler Dice Real-Time System MRQ (TP800, Takara Bio, Shiga, Japan) with SYBR premix Ex Taq II (Takara Bio). The following primers were used for real-time PCR: Zygo-F1 and Zygo-R1 for Rhizopus sp. and AspG and Asp2Aory for Aspergillus sp. (Table 1) [14,15]. Amplicon and genomic DNA of Rhizopus oryzae NBRC 4706 and Aspergillus luchuensis IFO 4308 were used as standards, respectively.

**Enzyme activity**

The sample (10 g) was mixed with 50 mL deionized water, homogenized (T25 digital ULTRA-TURRAX homogenizer, IKA Works GmbH, Staufen, Germany) at 11 000 rpm for 1 min, and subsequently centrifuged (Himac CF15RXII, Hitachi Koki, Ibaraki, Japan) at 4800 × g for 5 min. The supernatant was used for the α-amylase and protease assays. An α-amylase assay kit (Kikkoman Biochemifa, Tokyo, Japan) was used to measure α-amylase activity. Protease activity was determined as described in our previous study [5]. For the lipase assay, crude enzyme in the sample was extracted using a 0.1 M acetate buffer solution (pH 5.0) and ethanol, and the activity was determined using a Lipase Activity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA).

**Table 1 | Primers used in this study**

| Name    | Sequence (5'-3') | Reference          |
|---------|------------------|--------------------|
| Zygo-F1 | TTCAAAGGTCTGGG   | Francesconi et al., 2008 |
| Zygo-R1 | CAGTCTGGCTCCAA   | Francesconi et al., 2008 |
| AspG    | GCCAGCGAGTACAT    | Goebes et al., 2007 |
| AspAory | ACCCTCCTGGGCA     | Modified from Goebes et al., 2007 |

**Figure 2 | Growth of filamentous fungi during microbial fermentation.** The DNA copy number of each filamentous fungus was used as an indicator of growth. The solid line represents the growth of Aspergillus oryzae, and the dotted line represents the growth of Rhizopus oryzae.

**Table 2 | Enzyme activities in unfermented materials and MMF prepared with different activities of filamentous fungi (unit/g DW)**

|                   | Aspergillus oryzae | Rhizopus oryzae |
|-------------------|--------------------|-----------------|
|                   | Unfermented材料 | MMF-A           | Unfermented材料 | MMF-R           |
| α-Amylase         | 0.67 ± 0.06        | 138.68 ± 8.30*  | 0.54 ± 0.04     | 9.79 ± 0.85***  |
| Protease          | 358 ± 34           | 31 795 ± 3735*  | 741 ± 90        | 40 301 ± 4345*  |
| Lipase            | 741 ± 31           | 6835 ± 555*     | 793 ± 46        | 509 ± 341**     |

Data represent means ± standard deviations of three independent experiment results.

* Significantly different from unfermented materials by Student’s t-test (P < 0.05).

** Significantly different from MMF-A by Student’s t-test (P < 0.05).

DW, dry weight; MMF, Massa Medicata Fermentata.
### Table 3 | Volatile contents in unfermented materials and MMF prepared with different filamentous fungi

| Name                  | RI   | m/z   | Identification | Contents (µg/100 g DW) | Aspergillus oryzae | Rhizopus oryzae | Aspergillus oryzae | Rhizopus oryzae |
|-----------------------|------|-------|----------------|-------------------------|-------------------|-----------------|-------------------|-----------------|
|                       |      |       |                |                         | Unfermented      | MMF-A           | Unfermented      | MMF-R           |
| Terpenoids            |      |       |                |                         | materials        |                 | materials        |                 |
| 1,8-Cineole           | 1198 | 43    | MS,RI          | 5.48 ± 0.87             | 0.24 ± 0.09*     | 4.82 ± 1.92     | 0.23 ± 0.08*     |
| α-Limonene            | 1200 | 68    | MS,RI          | 1.24 ± 0.23             | 1.02 ± 0.07      | 1.48 ± 0.27     | 0.93 ± 0.30      |
| Geranylacetone        | 1842 | 69    | MS,RI          | 8.46 ± 0.42             | 1.86 ± 0.38*     | 9.82 ± 0.43     | 2.23 ± 0.37*     |
| Caryophyllene         | 1357 | 93    | MS,RI          | 1.13 ± 0.16             | 0.83 ± 0.07*     | 1.51 ± 0.16     | 1.96 ± 0.41**    |
| Camphor               | 1490 | 95    | MS,RI          | 62.71 ± 7.96            | 4.70 ± 0.40*     | 58.71 ± 8.07    | 7.34 ± 0.74***   |
| Aliphatic compounds   |      |       |                |                         |                   |                 |                   |                 |
| 2,3-Butanediol        | 1545 | 45    | MS,RI          | n.d.                    | 115 835 ± 47 378  | n.d.            | 118 167 ± 36 758 |
| Acetoin               | 1301 | 45    | MS,RI          | n.d.                    | 3035 ± 1988      | n.d.            | 6813 ± 5623      |
| Isoamyl alcohol       | 1193 | 55    | MS,RI          | 20.03 ± 8.07            | 14.55 ± 5.79     | 20.09 ± 2.31    | 42.88 ± 5.32***  |
| 1-Hexanol             | 1356 | 56    | MS,RI          | 9.80 ± 1.56             | 2.85 ± 0.48*     | 14.21 ± 4.40    | 4.86 ± 0.43***   |
| 1-Octanol             | 1536 | 56    | MS,RI          | 0.30 ± 0.02             | 0.36 ± 0.03      | 0.38 ± 0.03     | 0.19 ± 0.03***   |
| Hexanal               | 1085 | 56    | MS,RI          | 34.62 ± 9.77            | 8.65 ± 0.63*     | 35.41 ± 8.67    | 2.34 ± 1.12***   |
| 1-Octen-3-ol          | 1442 | 57    | MS,RI          | 1.60 ± 0.19             | 97.75 ± 10.63*   | 1.11 ± 0.18     | 1.17 ± 0.31**    |
| 2-Ethylhexanol        | 1478 | 57    | MS,RI          | 2.63 ± 0.36             | 0.49 ± 0.19*     | 1.99 ± 0.47     | 0.33 ± 0.11*     |
| 3-Octanone            | 1269 | 57    | MS,RI          | 0.22 ± 0.04             | 0.42 ± 0.05*     | 0.15 ± 0.03     | 0.15 ± 0.02**    |
| (E)-2-Octenal         | 1477 | 70    | MS,RI          | 1.54 ± 0.41             | 5.59 ± 0.96*     | 1.83 ± 0.47     | 1.08 ± 0.22**    |
| (E)-2-Nonenal         | 1511 | 70    | MS,RI          | 1.70 ± 0.24             | 2.03 ± 0.29      | 2.26 ± 0.15     | 2.69 ± 0.33      |
| 2-Pentylfuran         | 1231 | 81    | MS,RI          | 0.82 ± 0.08             | 1.42 ± 0.31*     | 0.79 ± 0.10     | 1.29 ± 0.22*     |
| γ-Nonalactone         | 2009 | 85    | MS,RI          | 22.46 ± 3.60            | 3.19 ± 1.06*     | 24.32 ± 5.48    | 2.49 ± 0.50*     |
| Ethyl palmitate       | 2218 | 88    | MS,RI          | 0.87 ± 0.76             | 4.28 ± 2.46      | 0.68 ± 0.84     | 6.61 ± 0.91*     |
| δ-Decalactone         | 2063 | 99    | MS,RI          | 0.19 ± 0.02             | 0.74 ± 0.10*     | 0.21 ± 0.08     | 0.79 ± 0.22*     |
| Aromatic compounds    |      |       |                |                         |                   |                 |                   |                 |
| 2-Phenylethanol       | 1893 | 91    | MS,RI          | 64.17 ± 19.17           | 71.05 ± 13.29    | 59.12 ± 1.23    | 150.68 ± 31.74***|
| Benzy1 alcohol        | 1858 | 108   | MS,RI          | 866.89 ± 81.11          | 17.54 ± 0.23*    | 820.49 ± 336.66 | 1969 ± 425***    |
| Benzaldehyde          | 1509 | 106   | MS,RI          | 86.95 ± 15.27           | 19.87 ± 6.89*    | 223.32 ± 184.33 | 25.81 ± 3.49     |
| 2,6-Dimethylpyrazine  | 1341 | 108   | MS,RI          | n.d.                    | n.d.             | 33.31 ± 17.41   | 14.48 ± 7.39     |
| 2,3-Dimethylpyrazine  | 1356 | 108   | MS,RI          | n.d.                    | n.d.             | n.d.            | 43.27 ± 15.90    |
| Trimethylpyrazine     | 1406 | 122   | MS,RI          | n.d.                    | n.d.             | n.d.            | 14.06 ± 11.38    |
| Tetramethylpyrazine   | 1466 | 136   | MS,RI          | n.d.                    | n.d.             | n.d.            | 14.06 ± 11.38    |
| Phenol                | 1905 | 94    | MS,RI          | 256.62 ± 90.29          | 102.68 ± 36.10   | 223.44 ± 48.65  | 270.42 ± 20.71** |
| Guaiacol              | 1782 | 124   | MS,RI          | n.d.                    | 21.48 ± 8.48     | n.d.            | 115.09 ± 3.93*** |
| 4-Vinylguaiacol       | 2136 | 150   | MS,RI          | 6.47 ± 1.51             | 197.32 ± 95.71*  | 6.69 ± 1.00     | 389.11 ± 195.94* |
| Butylated            | 1902 | 205   | MS,RI          | 0.07 ± 0.03             | 1.32 ± 0.21*     | 0.07 ± 0.03     | 1.58 ± 0.01*     |
| hydroxytoluene       |      |       |                |                         |                   |                 |                   |                 |
| Naphthalene           | 1724 | 128   | MS,RI          | 0.05 ± 0.01             | 0.03 ± 0.01      | 0.06 ± 0.00     | 0.03 ± 0.01*     |

Data represent means ± standard deviations of three independent experiment results.

* Significantly different from unfermented materials by Student’s t-test (P < 0.05).

** Significantly different from MMF-A by Student’s t-test (P < 0.05).

DW, dry weight; MMF, Massa Medicata Fermentata; MS, mass spectroscopy; n.d., not detected; RI, retention index.
Gas chromatography–mass spectrometry (GC–MS) analysis

The sample (10 g) was mixed with 50 mL deionized water, homogenized at 11 000 rpm for 1 min, and subsequently centrifuged at 4800 × g for 5 min. The volatile compounds in the supernatant were quantified using GC–MS (Agilent, 6890 N/5975B MSD, Agilent Technologies, Palo Alto, CA, USA) with stir bar sorptive extraction (GERSTEL TDS 3, GERSTEL CIS 4, GERSTEL K.K., Tokyo, Japan), as described previously [5]. The content of each compound was determined using the calibration curve obtained from the peak area of the known concentration of the compound standard.

Ferulic acid and free amino acid analysis

The sample (10 g) was mixed with 50 mL deionized water, homogenized at 11 000 rpm for 1 min, and subsequently centrifuged at 4800 × g for 5 min. The supernatant was filtered through a 0.45 μm cellulose acetate membrane filter (Toyo Roshi Kaisha Ltd., Tokyo, Japan) and used for analysis. Ferulic acid was quantified using an HPLC system equipped with a UV/VIS detector (SPD-20AV, Shimadzu, Kyoto, Japan) at a detection wavelength of 280 nm, as described in our previous study [5]. Free amino acids were quantified using an HPLC system equipped with a fluorescence detector (RF-10AXL, Shimadzu), as in our previous study [16].

**RESULTS**

Fungal growth during MMF production

The morphological changes in the raw materials of MMF are shown in Figure 1. Numerous yellowish spores were observed in MMF-A and long mycelia were observed in MMF-R. Quantitative PCR was used to evaluate fungal growth at the DNA level. DNA copies of both filamentous fungi rapidly increased 1000-fold in 24 h (Fig. 2). Although A. oryzae continued to grow from 24 to 48 h, R. oryzae did not grow after 24 h.

Enzyme activity in MMF

We measured the enzyme activity of MMF and unfermented materials (samples immediately after mixing the raw materials and conidia; Fig. 1). α-Amylase and protease activities were higher in both the MMF products than in unfermented materials (Table 2). Although lipase activity increased through fermentation with A. oryzae, it remained unchanged with R. oryzae.

Table 4 | Free amino acids in unfermented materials and MMF prepared with different filamentous fungi contents (mmol/g DW)

| Aspergillus oryzae | MMF-A | Rhizopus oryzae | MMF-R |
|-------------------|-------|----------------|-------|
| Aspartic acid     | 1.8 ± 0.1 | 13.6 ± 1.4* | 1.3 ± 0.3 | 6.2 ± 1.3*** |
| Threonine         | 2.3 ± 0.2 | 10.6 ± 1.3* | 2.0 ± 0.5 | 20.2 ± 6.7*** |
| Serine            | 0.8 ± 0.1 | 12.0 ± 1.5* | 0.4 ± 0.3 | 9.2 ± 1.5* |
| Glutamate         | 2.6 ± 0.2 | 43.1 ± 4.4* | 3.3 ± 1.2 | 14.2 ± 5.2*** |
| Proline           | 1.5 ± 0.1 | 16.2 ± 2.2* | 1.3 ± 0.1 | 10.6 ± 2.9* |
| Glycine           | 1.2 ± 0.1 | 11.0 ± 1.1* | 1.1 ± 0.2 | 8.6 ± 2.8* |
| Alanine           | 3.1 ± 0.2 | 17.5 ± 1.6* | 2.7 ± 0.2 | 21.0 ± 2.4* |
| Valine            | 1.1 ± 0.1 | 9.2 ± 1.1* | 1.1 ± 0.4 | 10.0 ± 3.0* |
| Methionine        | 0.4 ± 0.0 | 3.5 ± 0.4* | 0.3 ± 0.1 | 2.8 ± 0.3* |
| Isoleucine        | 0.6 ± 0.0 | 7.5 ± 0.9* | 0.5 ± 0.1 | 7.1 ± 1.7* |
| Leucine           | 1.2 ± 0.1 | 13.3 ± 1.6* | 1.0 ± 0.3 | 12.3 ± 2.1* |
| Tyrosine          | 0.5 ± 0.0 | 1.9 ± 0.3* | 0.5 ± 0.1 | 5.7 ± 0.4*** |
| Phenylalanine     | 1.7 ± 0.1 | 3.0 ± 0.3* | 1.5 ± 0.2 | 9.5 ± 1.0*** |
| Histidine         | 1.0 ± 0.0 | 7.3 ± 0.6* | 0.8 ± 0.0 | 8.4 ± 0.6* |
| Lysine            | 2.2 ± 0.1 | 15.2 ± 2.0* | 1.7 ± 0.9 | 10.6 ± 2.7* |
| Arginine          | 4.1 ± 0.3 | 18.6 ± 1.3* | 1.8 ± 0.9 | 13.5 ± 2.6*** |
| Sum               | 26.1 ± 0.9 | 203.4 ± 17.2* | 21.5 ± 1.4 | 172.9 ± 21.5* |

Data represent means ± standard deviations of three independent experiment results.

* Significantly different from unfermented materials by Student’s t-test (P < 0.05).

** Significantly different from MMF-A by Student’s t-test (P < 0.05).

DW, dry weight; MMF, Massa Medicata Fermentata.
oryzae. MMF-A showed significantly higher α-amylase and lipase activities ($P < 0.0001$) than MMF-R, and protease activity was higher in MMF-R than in MMF-A.

**Volatiles in MMF**

Thereafter, we attempted to elucidate the effects of fungal fermentation on the volatiles of MMF (Table 3). A total of 32 compounds were quantified in this study: nine alcohols, four aldehydes, four amines, six ketones, four phenols, and five other compounds. Unfermented materials contained more 1,8-cineole, 1-hexanol, 2-ethylhexanol, hexanal, camphor, geranylacetone, and γ-nonalactone than both types of MMF. Although no significant differences were noted, benzaldehyde was higher in unfermented materials. Meanwhile, 2,3-butanediol, acetoin, and guaiacol were detected in MMF only and not in unfermented materials. In addition, MMF contained more δ-decalactone, 4-vinylguaiacol, butylated hydroxytoluene, and 2-pentylfuran than unfermented materials. Although no significant differences were noted, ethyl palmitate was higher in MMF.

Between MMF-A and MMF-R, MMF-A contained more aliphatic compounds (such as 1-octen-3-ol, 1-octanol, hexanal, (E)-2-octenal, and 3-octanone) than MMF-R. Meanwhile, aromatic compounds (e.g., benzyl alcohol, β-phenylethyl alcohol, guaiacol, and phenol) were significantly higher in MMF-R ($P < 0.05$). Especially, the benzyl alcohol content was significantly decreased during MMF-A production, but increased during MMF-R production. Benzaldehyde and 4-vinylguaiacol were also higher in MMF-R. The content of ferulic acid, a precursor of guaiacol and 4-vinylguaiacol, was 287.1 and 577.9 nmol/g in MMF-A and MMF-R, respectively. Moreover, all pyrazines were specifically detected in MMF-R.

As higher alcohols and pyrazines are derived from free amino acids via microbial metabolism or chemical reactions, free amino acid content was also quantified (Table 4). The total amino acid content was 10 times higher in MMF than in unfermented materials. Although the sum of amino acids was not significantly different, several amino acids differed between MMF-A and MMF-R. MMF-R contained significantly higher amounts of tyrosine, phenylalanine, and threonine while MMF-A contained more aspartic acid, glutamate, and arginine ($P < 0.001$).

**DISCUSSION**

We investigated the enzyme activity and volatile properties of MMF prepared with *A. oryzae* or *R. oryzae* to elucidate the effects of microbial fermentation on the quality of MMF. We validated the fungal growth on raw materials of MMF as it has not been investigated whether these filamentous fungi can grow on raw materials of MMF during the fermentation process. Real-time PCR showed that both filamentous fungi rapidly grew on substrates and increased a 1000-fold in 24 h. We confirmed that microbial fermentation was conducted during MMF production. As rapid growth of filamentous fungi would contribute to the prevention of undesired microbial contamination, the inoculation of pure cultures of conidia on raw materials would be effective for the stable production of MMF.

MMF-A showed higher α-amylase and lipase activities than MMF-R. MMF-R showed marginally higher protease activity than MMF-A. These results are consistent with those of a previous study in which soy bean-koji prepared with *A. oryzae* showed extremely high α-amylase activity but lower protease activity than that prepared with *R. oryzae* [17]. Although *R. oryzae* has been reported to produce lipase [18], lipase activity was not increased in MMF-R. As *R. oryzae* reportedly produces lipase after 48 h of incubation [18], it is possible that lipase of MMF-R would increase by extending the fermentation duration. Microbial differences could affect the enzymatic activity of MMF.

The volatiles changed drastically owing to microbial fermentation. Terpenoids and benzaldehyde were higher in unfermented materials than in MMF. We expected that terpenoids were increased via hydrolyzation of glycosides in plant materials during fermentation; however, our results showed that terpenoids were not increased by MMF production. Sweet wormwood contains essential oils, including camphor and 1,8-cineole, and the apricot kernel is known to contain benzaldehyde [19,20]. Therefore, these compounds were considered to be derived from such plant materials and would be metabolized or vaporized by the fermentation and drying process. Meanwhile, both types of MMF contained considerable amounts of 2,3-butanediol and acetoin, which were not detected in unfermented materials. These compounds were synthesized by *Aspergillus* sp. and *Rhizopus* sp. [21,22]. As the contents of 2,3-butanediol and acetoin were considerable, these compounds were possible indicators of microbial fermentation of commercial MMF.

The amount of C8 compounds was higher in MMF-A than in MMF-R. In particular, the 1-octen-3-ol level in MMF-A was 90-fold higher than in unfermented materials and MMF-R. 1-Octen-3-ol is a characteristic volatile compound produced by *Aspergillus* sp. and is synthesized from linoleic acid by fatty acid oxygenase [23]. Zhao *et al.* recently reported that the fatty acid oxygenase activity of *A. luchuensis* is significantly higher than that of *R. oryzae* [24]. Therefore, the higher activity of fatty acid oxygenase in *A. oryzae* would contribute to a higher amount of 1-octen-3-ol in MMF-A. (E)-2-Octenal is an oxidative degradation product of fatty acids [25], and 1-octanol and 3-octanone have also been reported to be produced by *Aspergillus* spp. [26]. As MMF-A showed increased lipase activity, more fatty acids appeared to be supplied during fermentation and subsequently oxidized to C8 compounds.

In contrast, aromatic compounds were higher in MMF-R than in MMF-A. In addition, MMF-R contained...
various compounds at high concentrations (>100 μg/100 g). Phenolics such as guaiacol and 4-vinylguaiacol were higher in MMF-R, and were reported to be derived from ferulic acid by a decarboxylation reaction [9]. As MMF-R contained more ferulic acid than MMF-A, the higher precursor content accelerated the formation of guaiacol and 4-vinylguaiacol. Ferulic acid and 4-vinylguaiacol have been reported to have anti-inflammatory effects, and these compounds contribute to the medicinal properties of MMF [9].

Amino acid-related compounds such as β-phenylethyl alcohol, isoamyl alcohol, and pyrazines were also higher in MMF-R than in MMF-A. β-Phenylethyl alcohol and isoamyl alcohol are known to be biosynthesized in yeast from phenylalanine and leucine via the Ehrlich pathway, respectively. The biosynthesis of these higher alcohols has also been suggested to occur in R. oryzae [27,28]. Pyrazines were reported to be derived from threonine, serine, and phenylalanine by the Maillard reaction [29,30]. The increased protease activity in MMF-R seemed to accelerate the formation of such volatiles. When precursor amino acids in MMF were compared, phenylalanine and threonine levels were higher in MMF-R than MMF-A; however, leucine and serine levels were not different between MMF-A and MMF-R. Apparently, amino acids derived from protease were continuously metabolized during fermentation and partially changed to volatiles.

MMF-A and MMF-R showed some similarities with the commercial MMF described in our previous study [5]. The commercial MMF in which Aspergillus sp. was predominant, showed increased enzymatic activities and contained more 1-octen-3-ol and 2-octanone. The commercial MMF in which Rhizopus sp. was detected contained more benzyl alcohol and benzaldehyde. In addition, 4-vinylguaiacol and pyrazines were detected only in commercial MMF in which Rhizopus sp. was detected. As pyrazines were specifically contained in MMF-R, pyrazines could be useful markers for distinguishing fungal species in MMF. Our present work revealed that the characteristics of volatiles in commercial MMF were partially due to the presence of Aspergillus or Rhizopus. However, the differences in all volatiles of commercial MMF could not be explained by this study. For example, phenethyl alcohol content was not higher in commercial MMF with Rhizopus sp. In addition, some commercial MMF lacked 2,3-butanediol and acetoin. As Chinese MMF contains various fungi and bacteria other than filamentous fungi [5], differences in microbial content can affect the volatiles in MMF. The effects of symbiotic microorganisms and their interactions should be investigated in future studies.

In this study, we investigated the effects of microbial fermentation on the quality of MMF. Microbial fermentation with A. oryzae or R. oryzae contributed to increased enzyme activity and volatiles, which are metabolites of these filamentous fungi. Meanwhile, plant-derived essential oil components decreased during fermentation. MMF-A showed higher α-amylase and lipase activities and contained more C8 compounds. Alternatively, MMF-R showed marginally higher protease activity and contained more aromatic compounds. The quality of MMF could to a considerable extent be affected by the microbes used, and microbial control in the production of commercial MMF was proposed to be important in this study. Although we focused on the effects of fungal fermentation on enzymatic activity, microbial difference alone could not explain the variation in the quality of commercial MMF. The effects of symbiotic microorganisms and their interactions need to be investigated in the future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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