Phytochemical properties and functional characteristics of wild turmeric (Curcuma aromatica) fermented with Rhizopus oligosporus

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ABSTRACT

This study investigated the effect of solid-state fermentation of wild turmeric (Curcuma aromatica) with Rhizopus oligosporus, a common fungus found in fermented soy temeph, on phytochemical and biological properties. Ultra-performance liquid chromatography–tandem mass spectrometry showed that fermented wild turmeric has higher concentrations of curcumin, demethoxycurcumin, bisdemethoxycurcumin, phenolic compounds and total flavonoid-curcuminoinds after fermentation for 1-, 3-, and 5-day relative to non-fermented turmeric. The L-carnitine content reached 242 μg g⁻¹ extract after fermentation for 7-day. Wild turmeric had 1.47- and 2.25-fold increases in ORAC and FRAP, respectively, after 3-day fermentation. The inhibitory effects of fermented wild turmeric on lipid accumulation from 3T3-L1 cells, nitric oxide production from lipopolysaccharide-stimulated RAW264.7 murine macrophages, and melanin formation by B16F10 mouse melanoma cells with α-MSH increased 1.08-, 1.44-, and 1.52-fold, respectively, after 3-day fermentation. Based on these results, fermented wild turmeric product can be used as a functional ingredient in the cosmeceutical and nutraceutical industries.

1. Introduction

Wild turmeric (Curcuma aromatica) of the genus Curcuma in family Zingiberaceae is widely planted in China, Japan, and India (Xiang et al., 2017). C. aromatica is characterized by a bright yellow aromatic rhizome and camphoraceous odor. It is commonly used as a coloring and flavoring agent, as well as in traditional medicines (Rajkumari and Sanatombi, 2018). The rhizomes of wild turmeric contain curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) and essential oils including xanthorrhizol, α-curcumene, di-γ-α-cedrene, turmerone, α-turmerone, zingiberene, β-sesquiphellandrene, germacrone, 8,9-dehydro-9-formyl-cycloisoolongifolene, furanodienone, and zederone (Kulyal et al., 2021) that contribute to its anti-inflammatory, anticancer, antibacterial, antisebaceous, anti-inflammatory, and antioxidant activities (Xiang et al., 2018; Xiang et al., 2017). Although C. aromatica is one of the most common Curcuma species and is widely used along with Curcuma longa (turmeric), limited literature is available related to enhancement of its food value, nutritional composition, and health benefits. In addition, the strong flavor and taste of turmeric limit its use in food industrial applications and decrease palatability to consumers (Kim et al., 2016).

Solid-state fermentation (SSF) has gained attention in recent years from biotech industries including in bioremediation; biofuels; production of lipids, flavors, and aromas for food; and production of bioactive compounds (Sala, Barrena, Artola, & Sanchez, 2019). SSF involves the growth of microorganisms on a solid substrate with a low moisture content (Holker, Hofer, & Lenz, 2004). Fungi are the main group of microorganisms used for SSF (Sala et al., 2019). SSF has several advantages over submerged fermentation including high volumetric productivity, product concentration, simplicity, and low sterilization cost (Jimenez-Quero, Pollet, Averous, & Philap, 2020). SSF with Trichoderma spp. and Aspergillus oryzae has been used for the fermentation of
turmeric (C. longa L.) (Kim et al., 2014; Mohamed, Saleh, Kabli, & Al-Garni, 2016). Compared to non-fermented turmeric, turmeric fermented with Trichoderma spp. showed increases in water solubility, total phenolic content, and antioxidant and antibacterial activities (Mohamed et al., 2016). Turmeric fermented with A. oryzae was effective at preventing CCl₄-induced hepatic damage in rats; reducing white adipose tissue weight, serum triglyceride, and cholesterol in rats with high-fat diet-induced obesity; and showed improvement of the strong taste and bitterness, thereby increasing consumer acceptance (Kim et al., 2016; Kim et al., 2014).

*Rhizopus oligosporus* is a common fungus used for fermentation of soy tempeh. It produces various enzymes including amylase, protease, glucoamylase, and lipase, as well as carbohydrate-cleaving enzymes including xylanase, β-glucoronidase, and β-glucosidase to synthesize L-carnitine, γ-aminobutyric acid (GABA), and polyphenols during fermentation (Hur, Nguyen, Park, Kim, & Kim, 2018; Lee et al., 2020; Park et al., 2018). Moreover, coffee fermented via SSF using *R. oligosporus* had improved aroma and sweetness (Lee, Cheong, Curran, Yu, & Liu, 2016).

This study aims to analyze the physicochemical properties and functional characteristics of *R. oligosporus*-fermented wild turmeric extract. Wild turmeric was subjected to solid state fermentation, L-carnitine, vanillin, ferulic acid, curcumin, demethoxycurcumin, and bisdemethoxycurcumin in the extracts were identified by ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS) and the volatile compounds responsible for the sensory attributes of fermented wild turmeric were characterized by gas chromatography–mass spectrometry (GC-MS). The antioxidant properties of wild turmeric were determined by Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAC) assays. In addition, anti-inflammatory activity in RAW264.7 murine macrophages, anti-melanogenic activity in B16F10 mouse melanoma cells, and anti-lipid accumulation activity in mouse 3T3-L1 cells was evaluated.

2. Materials and methods

2.1. Materials and reagents

Wild turmeric powder was purchased from Good Herb Inc. (Seoul, Korea). Curcumin, demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), and L-carnitine were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Folin–Ciocalteau reagent, 2,2'-azobis (2-methylpropionimidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroaneman-2-carboxylic acid (Trolox), lipopolysaccharides (LPS), indomethacin, vanillin, ferulic acid, alpha-melanocyte-stimulating hormone (α-MSH), dimethyl sulfoxide (DMSO), quercetin (Q), and gallic acid (GA), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein was purchased from Alfa Aesar (Haverhill, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum (BCS), and fetal bovine serum (FBS) were purchased from Gene Depot (Barker, TX, USA), and penicillin and streptomycin (PS) were purchased from Invitrogen (Carlsbad, CA, USA). Ez-CyTox solution for cell viability assays was purchased from Daeil Lab Service (Seoul, Korea).

2.2. Solid-state fermentation of wild turmeric with *R. oligosporus*

2.2.1. Preparation of the fermented wild turmeric

*R. oligosporus* KCCM 11948P was obtained from our previous study (Park et al., 2017) and cultured on potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 30 °C for 5 days to obtain spores. Turmeric powder (10 g) was placed in a tetragonal stainless-steel container (100 × 40 mm), and soaked in water at a ratio of 1:2 (w/w) for 12 h at 22 °C. Soaked turmeric was autoclaved (JSAT-105; JSR, Gongju, Korea) at 121 °C for 20 min. Autoclaved turmeric was cooled to ambient temperature (22 °C) and inoculated with 10% (v/v) *R. oligosporus* spore suspension (10⁶ spores mL⁻¹). After covering the container with wrapping paper, turmeric was incubated at 30 °C and 70–95% relative humidity for 1–3, 5–9, and 7-day. Experiments were carried out with three replicates for each incubation period. Finally, fermented wild turmeric was frozen at – 80 °C and lyophilized at 0 °C and 10 Pa (Eyela FD-550; Rikakikai Co., Tokyo, Japan).

2.2.2. Sample extraction

A 1-g sample of wild turmeric or fermented wild turmeric was extracted into 10 mL of 70% (v/v) ethanol through shaking for 1 h at 150 rpm and 30 °C, followed by sonication (VXC 130 Ultrasonic Processor, Sonics and Materials, Inc., Newtown, CT, USA; 3 min sonication amplitude of 40%, output wattage of 20, 5 repeats, on ice). The supernatant was obtained through centrifugation (9600 × g for 15 min at 4 °C). The pellets were subjected to the process described above three times, and the supernatant was filtered using Whatman No. 1 filter paper (Piscataway, NJ, USA). Next, ethanol was removed by evaporation (Heidolph Instruments, Schwabach, Germany) and lyophilized at 0 °C and 10 Pa for further analysis. The extraction yield was calculated as described in our previous report (Hur et al., 2018), as follows:

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\text{Extraction yield} (\%) = \frac{\text{Extract mass (g)}}{\text{Wild turmeric mass (g)}} \times 100
\]

2.3. Phytochemical analysis of fermented wild turmeric

2.3.1. Total phenolic content (TPC) and total flavonoid-curtainoid content (TFCC) analysis

The TPC of wild turmeric and fermented wild turmeric was determined using the Folin–Ciocalteau method with gallic acid as the standard. TPC values are presented as mM gallic acid equivalent g⁻¹ (mM GAE g⁻¹) (Hur et al., 2018). TFCC analysis was performed using the aluminum chloride colorimetric method (Chang, Yang, Wen, & Chen, 2002) with quercetin (0–330.9 μM) as the standard. TFCC was expressed as mM quercetin equivalent g⁻¹ (mM QE g⁻¹).

2.3.2. L-carnitine analysis

Ten milligrams of non- or fermented wild turmeric extracted powder was dissolved in DMSO, diluted with methanol, and filtered through a 0.2 μm pore-size membrane syringe filter (Pall, NY, USA). A 1 μL sample was injected into the UPLC-MS system (Acquity H-Class system with a QDa detector; Waters, Milford, MA, USA) and separated using an Acquity UPLC Beh HILIC column (1.7 μm, 2.1 × 100 mm; Waters) at 40 °C. The elution gradient for L-carnitine analysis was previously reported (Lee et al., 2020). Positive electrospray ionization (ESI) was controlled with selected ion recording (SIR) (m/z 162). The capillary energy and cone voltage were 1.5 kV and 10 V, respectively. A calibration curve was prepared using various l-carnitine concentrations (0.02–2 μg mL⁻¹) (Table S1).

2.3.3. Curcinoids, vanillin, and ferulic acid analysis

A 1 μL sample was injected into the UPLC-MS system and separated using an Acquity UPLC Beh C₁₈ column (1.7 μm, 2.1 × 150 mm; Waters) at 40 °C. The mobile phase for analysis was composed of acetonitrile containing 0.1% (v/v) formic acid (solvent A) and water containing 0.1% (v/v) formic acid (solvent B). For analysis of curcinoids, the initial elution gradient was 5% A, with increases to 50% A at 1.0 min, 70% A at 5.0 min, and 100% A at 6.5 min, a decrease to 5% A at 7.1 min, and a hold at that level until 12.0 min for equilibrium, with a flow rate of 0.3 min mL⁻¹. For analyses of vanillin and ferulic acid, the initial elution gradient was 5% A, which increased to 10% A at 0.5 min, 15% A at 2.1 min, 23% A at 10.0 min, 50% A at 12.0 min, 60% A at 15.0 min, and 100% A at 15.1 min, then decreased to 5% A at 16.1 min, and was
maintained at that level until 25.0 min for an equilibration step, with a flow rate of 0.3 min mL$^{-1}$. Positive ESI was controlled with SIR (m/z 369.27 for curcumin, m/z 339.22 for demethoxycurcumin, m/z 309.19 for bisdemethoxycurcumin, m/z 195.13 for ferulic acid, and m/z 153.11 for vanillin). The capillary voltage and cone voltage used for assessment of curcuminoids were 1.3 kV and 10 V and 1.5 kV and 10 V for vanillin and ferulic acid, respectively. A calibration curve was prepared using various concentration of curcuminoids (0.02–2 μg mL$^{-1}$), vanillin (0.08–20 μg mL$^{-1}$), and ferulic acid (0.04–2 μg mL$^{-1}$) (Table S1).

2.3.4. Analysis of volatile compounds in fermented wild turmeric

Volatile compounds in non-fermented and fermented wild turmeric were extracted through headspace-solid-phase microextraction (HS-SPME). A 3 g sample of wild turmeric powder or fermented wild turmeric was weighed into a 20 mL screw-capped headspace vial and subjected to HS-SPME at 60 °C for 30 min with agitation. Divinylbenzene/carboxen/polydimethylsiloxane (50/30 μm DVB/CAR/PDMS) was used. The volatile components of wild turmeric and fermented wild turmeric were assessed in a 7890 N GC coupled to a 5975C inert MSD Mass Detector (Agilent Technologies, Palo Alto, CA, USA). The SPME fiber was thermally desorbed into an HP-5MS column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent Technologies) in splitless mode. Helium was used as the carrier gas with the flow rate set to 1.5 mL min$^{-1}$. The GC oven temperature was maintained at 50 °C for 3 min, increased to 150 °C at a rate of 5 °C min$^{-1}$, to 200 °C at a rate of 10 °C min$^{-1}$, and to 280 °C at a rate of 50 °C min$^{-1}$, and then held at that temperature for 3 min. The range of mass detection was m/z 33 to 500. Volatiles were identified through comparison of their retention times and mass spectra with those of standards.

2.4. Antioxidant activity

2.4.1. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay of wild turmeric and fermented wild turmeric was conducted according to the method reported by Ou, Hampsch-Woodill, & Prior (2001) with Trolox as a standard, fluorescein as a probe, and AAPH to induce radical formation. The fluorescence of the reaction was read every 3 min ($\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 538$ nm) for up to 90 min at constant 37 °C with the SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The net area under the curve (AUC) was estimated by subtracting the AUC of the blank from the AUC of the reacted sample. Antioxidant activity was expressed as μM Trolox equivalent g$^{-1}$ (μM TE g$^{-1}$).

2.4.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assays of wild turmeric and fermented wild turmeric were conducted according to our previous report (Lee et al., 2020). A 20 μL sample of wild turmeric, fermented wild turmeric, or Trolox (1 – 40 μM) was mixed with 180 μL FRAP solution containing 0.3 M sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine, and 20 mM ferric chloride hexahydrate solution at a 1:10:1 (v/v/v) ratio. After a 30 min incubation in the dark, the reaction mixture was measured at 593 nm using the SpectraMax M3 microplate reader. FRAP was expressed as μM Fe$^{2+}$ g$^{-1}$.

2.5. Viability assay

Mouse 3T3-L1 cells, RAW264.7 murine macrophages, and B16F10 mouse melanoma cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Mouse 3T3-L1 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin at 37 °C in 5% CO$_2$. RAW264.7 murine macrophage cells and B16F10 mouse melanoma cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin at 37 °C under 5% CO$_2$. Cells grown in a 96-well plate at 2 × 10$^4$ cells/well were incubated for 24 h at 37 °C under 5% CO$_2$. Next, various concentrations of wild turmeric or fermented wild turmeric (12.5–200 μg mL$^{-1}$) were added to the wells of a 96-well plate and incubated at 37 °C for 24 h. Subsequently, 90 μL of culture medium mixed with 10 μL of Ez-CyTox solution were added and incubated for 1 h at 37 °C, and cell viability was measured at 450 nm using a SpectraMax M3 microplate reader. Cell viability was calculated relative to the control.

2.6. Anti-lipid accumulation test

Mouse 3T3-L1 cells grown in a six-well plate at 1 × 10$^5$ cells/well were incubated at 37 °C until 100% confluence. Two days after reaching confluence, differentiation was induced by replacing the growth medium with differentiation medium (DMEM, 10% FBS, 10 μg mL$^{-1}$ insulin, 500 μg 3-isobutyl-1-methylxanthine, and 1 μg dexamethasone) in the presence or absence of 50 μg mL$^{-1}$ samples (wild turmeric and fermented wild turmeric) (Kim et al., 2021). After 2 days, this induction medium was replaced with DMEM supplemented with 10% FBS and 100 U mL$^{-1}$ penicillin and streptomycin. After 7 days of differentiation, differentiated 3T3-L1 cells were fixed with 3.7% (v/v) formaldehyde for 30 min, and then removed. The fixed cells were stained with Oil red O staining solution for 15 min in the dark. Lipid droplets within the differentiated 3T3-L1 cells were visualized with a Zeiss Observer Z1 microscope (Carl Zeiss, Jena, Germany) and photographed with an AxioCam HRc camera. For quantitative analysis, Oil Red O staining was performed through elution with isopropanol and absorbance was measured at 490 nm using a SpectraMax M3 microplate reader. The results of lipid accumulation were expressed as percentages relative to the control.

2.7. Measurement of nitric oxide production

RAW264.7 murine macrophages were seeded in a 96-well plate at 2 × 10$^4$ cells/well and cultured for 24 h at 37 °C. The cells were treated with 25 μg mL$^{-1}$ sample and 1 μg LPS/mL at 37 °C for 24 h. Cells treated with 1 μg mL$^{-1}$ LPS and 100 μM indomethacin were used as the control. Then, 80 μL of culture supernatant was mixed with the same volume of Griess reagent, held at room temperature for 20 min, then measured for absorbance at 540 nm (Lee et al., 2020; Xu et al., 2021).

2.8. Anti-melanogenesis test

B16F10 mouse melanoma cells were seeded into a six-well plate at 1 × 10$^5$ cells/well and cultured for 24 h at 37 °C. Then, the cells were treated with 100 nM a-MSH and 50 μg mL$^{-1}$ wild turmeric or fermented wild turmeric for 72 h. Next, the cells were trypsinized and centrifuged at 1500 × g for 3 min. The cell pellets were solubilized in 1 M NaOH containing 10% (v/v) DMSO and melted at 80 °C for 30 min. Absorbance was measured at 405 nm using a SpectraMax M3 microplate reader.

2.9. Statistical analysis

All experiments were performed in triplicate, and all data are presented as mean ± standard deviation. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and the Pearson correlation test in SPSS software. Values were considered significant at $p < 0.05$. Principal component analysis (PCA) based on a correlation matrix was conducted to differentiate the effects of fermented wild turmeric using SigmaPlot v14.5 (Systat Software, San Diego, CA, USA).
Table 1
Phytochemical properties and antioxidant activities of fermented wild turmeric.

| Fermented turmeric (day) | \(\mu g \, g^{-1}\) extract | Curcumin | DMC | BDMC | Vanillic acid | ORAC | FRAP | TPC | TFCC |
|--------------------------|-----------------------------|---------|-----|------|--------------|------|------|-----|------|
| 0                        | ND                          | 4560±56 | 7b  | 29b  | 34b          | 533±34 | 1010±67 | 214.8±3.0 | 397±8.8 | 56.6±6.1 |
| 1                        | ND                          | 5313±45 | 12a | 36a  | 2a           | 576±6  | 1174±87 | 263.4±11.3 | 444±8.8  | 90.7±3.9 |
| 3                        | 26.6±1.4                   | 4660±104 | 37b | 9b   | 47±1        | 492±10 | 1471±47 | 336.4±11.9 | 584±18.4 | 127.2±5.6 |
| 5                        | 117±0.5                    | 4813±83 | 32b | 36m  | 57±5b       | 546±19  | 1423±98 | 286.2±6.1 | 538±10 | 111.1±0.7 |
| 7                        | 242±13.4                   | 4513±108 | 35b | 14a  | 44±4b       | 683±13  | 1414±67 | 210.5±4.9 | 461±7.8 | 69.5±6.5 |

DMC: demethoxycurcumin; BDMC: bisdemethoxycurcumin; ORAC: Oxygen Radical Absorbance Capacity; FRAP: Ferric Reducing Antioxidant Power; TPC: total phenolic content; TFCC: total flavonoid-curcuminoid content. ND: non-detected. All experiments were carried out in triplicate, and all the data were expressed as the mean ± standard deviation. The different letters of the alphabet in each column represent significant differences (p < 0.05).

3. Results and discussion

3.1. Influence of solid-state fermentation on phytochemical components of wild turmeric

3.1.1. Total phenol content and total flavonoid-curcuminoid content

To enhance the functionality, flavor, and nutritional value of wild turmeric, we fermented wild turmeric using \(R. \text{ oligosporus}\). After fermentation, the extraction yield, TPC, and TFCC of extracted wild turmeric, as well as those of fermented wild turmeric after 1-, 3-, 5-, and 3.1-day fermentation, were determined. The extraction yields of non-fermented, and 1-, 3-, 5-, and 7-day fermented wild turmeric were 18.2%, 17.5%, 19.4%, 17.7%, and 15.9%, respectively. As shown in Table 1, the TPC increased from 397 ± 8.8 mM GAE g⁻¹ in non-fermented wild turmeric to 584 ± 18 mM GAE g⁻¹ after 3-day fermentation, then decreased to 538 ± 10 and 461 ± 7.6 mM GAE g⁻¹ after 5-day and 7-day fermentation, respectively. The TFCC of wild turmeric increased from 56.6 ± 6.1 mM QE g⁻¹ to 127.2 ± 5.6 mM QE g⁻¹ after 3-day fermentation, and then gradually decreased to 69.5 ± 6.5 mM QE g⁻¹ after 7-day fermentation (Table 1). Curcuminoids are classified as diarylheptanoids and composed of two aromatic \(o\)-methoxy phenol groups linked with a seven-carbon chain (Priyadarsini, 2013). To date, over 300 diarylheptanoids in the \(Zingiberaceae\) and related families have been reported. Although, curcumin, DMC, and BDMC are the main curcuminoids in wild turmeric, several flavonoids are found in turmeric, such as luteolin-7-O-β-D-glucoside, 7-methoxyapigenin-6-C-glucoside (Shabana, Shab, Taha, Mahdy, & Mahmoud, 2015) and hydrocurcumin (Shabana, Shab, Taha, Mahdy, & Mahmoud, 2015). Thus, the aluminum chloride colorimetric method was used for the determination of both flavonoid and curcuminoid contents in this study. The TPC and TFCC of the fermented wild turmeric were enhanced by 1.12-1.47-fold and 1.23-2.25-fold compared to non-fermented wild turmeric. The highest TPC and TFCC of fermented wild turmeric were obtained after 3-day fermentation. Previous studies have reported that SSF caused increases of TPC from 41 to 80 mg GAE kg⁻¹ in quinoa after 5-day fermentation with \(R. \text{ oligosporus}\) (Hur et al., 2018), from 2.84 mM GAE g⁻¹ to 5.65 mM GAE g⁻¹ in wild ginseng after 7-day fermentation with \(R. \text{ oligosporus}\) (Lee et al., 2020), and from 2.5 to 23.3 mg GAE g⁻¹ in turmeric fermented with \(Chromobacterium\) spp. (Mohamed et al., 2016). Hur et al. (2018) showed that 5-day SSF of quinoa with \(R. \text{ oligosporus}\) increased the total flavonoid content by up to 1.46-fold relative to non-fermented quinoa. The increases of TPC and TFCC in wild turmeric fermented with \(R. \text{ oligosporus}\) could lead to production of carbohydrate-cleaving enzymes such as \(a\)-amylase, \(\beta\)-glucuronidase, and \(\beta\)-glucosidase, which degrade the cell wall matrix and generate polyphenols from carbohydrate-conjugated phenolic compounds during fermentation (Huynh et al., 2014; McCue & Shetty, 2003).

3.1.2. \(\iota\)-carnitine

\(\iota\)-carnitine (3-hydroxy-4-N-trimethylamino butyrate) is a quaternary amine that plays a crucial role in mitochondrial \(\beta\)-oxidation of fatty acids by transporting long-chain fatty acids across the mitochondrial membrane and buffering the free CoA/acyt-CoA ratio. \(\iota\)-carnitine is synthesized from lysine and methionine in the brain, liver, and kidney (approximately 25%) and obtained via dietary intake (approximately 75%) (Fielding, Riede, Lugo, & Bellamine, 2018). The primary dietary sources of \(\iota\)-carnitine are red meat (140-190 mg/100 g) and milk, while plant-derived foods contain insignificant quantities of \(\iota\)-carnitine (Fielding et al., 2018). As shown in Table 1, \(\iota\)-carnitine was not detected in wild turmeric or 1-day fermented wild turmeric. \(\iota\)-carnitine (26.6 \(\mu g\) g⁻¹) in wild turmeric was first detected after 3-day fermentation. Subsequently, it increased gradually with fermentation time (Table 1). The highest level of \(\iota\)-carnitine (242 \(\mu g\) g⁻¹) was obtained after 7-day fermentation with \(R. \text{ oligosporus}\) (Table 1). SSF with \(R. \text{ oligosporus}\) led to an increase in \(\iota\)-carnitine content from 0.13 to 3.15 \(\mu g\) g⁻¹ in quinoa after 3-day fermentation (Hur et al., 2018) and levels up to 630 \(\mu g\) g⁻¹ in wild ginseng after 14-day fermentation (Lee et al., 2020). \(R. \text{ oligosporus}\) hydrolyzes proteins into amino acids and small peptides (Handoyo & Morita, 2006) and synthesizes \(\iota\)-carnitine from lysine and methionine through four enzymatic reactions (Rousta, Ferreira, & Taherzadeh, 2021).

3.1.3. Curcumin, demethoxycurcumin, bisdemethoxycurcumin, vanillin, and ferulic acid

Curcuminoids such as curcumin, DMC, and BDMC are key bioactive compounds found in the genus \(Curcuma\) that contribute to its anti-inflammatory, anti-obesity, antioxidiant, antimutagenic, anticancer, antimicrobial, antiarthritic, neuroprotective, and tyrosinase inhibitory activities (Anand et al., 2008). Thus, the effects of fermentation time on curcuminoids in wild turmeric were investigated. As shown in Table 1,
All experiments were carried out in triplicate, and all the data were expressed as the mean ± standard deviation.

### 3.1.4. Volatile compounds

The volatile compound composition of wild turmeric is responsible not only for its aromatic flavor and odor but also its biological activities including antioxidant, anti-inflammatory, and anti-bacterial activities (Zhang et al., 2017). Table 2 and Figure S1 shows the volatile compounds found in non-fermented wild turmeric and in wild turmeric after 1-, 3-, 5-, and 7-day fermentation. In total, 16 volatile compounds were identified in fermented wild turmeric. The major compounds in non-fermented and fermented wild turmeric were attributed to the presence of phenolic compounds, flavonoid compounds, 1-carminite, and volatile oil compounds (Galain, 2006; Xiang et al., 2017). Therefore, increases or decreases of TPC, TFCC, 1-carminite, and volatile compounds are related to increases or decreases in the antioxidant activity of non-fermented and fermented wild turmeric.

### 3.3. Relationship between fermentation time and major components of wild turmeric

PCA was used to determine the effects of wild turmeric fermentation
time based on qualitative indicators (TPC, TFCC, curcumin, DMC, BDMC, ferulic acid, total phenolic content, total flavonoid-curtuminoid content, and antioxidant activities (ORAC and FRAP) of non- and fermented wild turmeric at different fermentation time: (A) PCA score plot (PC2 versus PC1) and (B) PCA biplot (PC2 against PC1).

Fig. 1. Variate statistical analyses of L-carnitine, curcumin, demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), vanillin, ferulic acid, total phenolic content, total flavonoid-curtuminoid content, and antioxidant activities (ORAC and FRAP) of non- and fermented wild turmeric at different fermentation time: (A) PCA score plot (PC2 versus PC1) and (B) PCA biplot (PC2 against PC1).

Fig. 2. Cell viability (A), lipid-droplets accumulation inhibition (B), and lipid accumulation inhibition (C) of non-fermented wild turmeric (NF) and fermented wild turmeric by R. oligosporus turmeric after 1-, 3-, 5-, and 7-day on 3T3-L1 cells. Data are means ± standard deviation (SD) of three independent experiments.

Fig. 1B indicates how the selected qualitative indicators were used to discriminate among wild turmeric fermentation times, with the length and direction of each variable (eigenvector) showing its relative contribution to the quality indicator. In PC1, 1-day fermented and non-fermented wild turmeric were characterized by high positive
coefficients for curcumin (0.919), DMC (0.24), and BDMC (0.156). A high concentration of curcuminoid clearly distinguished fermentation periods of wild turmeric. The direction of the eigenvectors and negative coefficients for vanillin (−0.386) are additional distinct characteristics of non-fermented wild turmeric.

3.4. Inhibition of lipid accumulation by fermented wild turmeric

In this study, the anti-lipid accumulation properties of wild turmeric and fermented wild turmeric were investigated based on inhibition of lipid droplet formation in 3T3-L1 cells. First, the cytotoxicity to 3T3-L1 cells of non-fermented and 1-, 3-, 5-, and 7-day fermented wild turmeric was assessed. The viability of 3T3-L1 cells treated with non-fermented or 1-, 3-, 5-, and 7-day fermented wild turmeric relative to untreated cells is shown in Fig. 2A. At 50 µg mL\(^{-1}\), cell viability was over 90%, and therefore we selected 50 µg mL\(^{-1}\) non-fermented or fermented wild turmeric for the anti-lipid accumulation test. The inhibitory effects of non-fermented and fermented wild turmeric on lipid accumulation were examined using Oil Red O staining. Fig. 2B shows lipid droplets in untreated control 3T3-L1 cells, as well as cells treated with non-fermented and 1-, 3-, 5-, and 7-day fermented wild turmeric on 7-day cell differentiation. As shown in Fig. 2B, cells treated with non-fermented and fermented wild turmeric had lower numbers of lipid droplets than control cells, which were not treated with wild turmeric. Seven-day lipid accumulation rates in 3T3-L1 cells in the control, non-fermented wild turmeric, and 1-, 3-, 5-, and 7-day fermented wild turmeric-treated groups were 100%, 67.0 ± 4.6%, 71.5 ± 3.5%, 61.8 ± 3.3%, 58.8 ± 8.8%, and 60.2 ± 6.5%, respectively (Fig. 2C). Our results are in agreement with previous research showing that the ethyl acetate fraction of C. longa root extract inhibited lipid accumulation in 3T3-L1 cells (Lee et al., 2010). The mechanism through which the ethyl acetate fraction obtained from turmeric inhibits lipid accumulation is via suppression of GLUT4 expression and stimulation of lipolysis, which involves induction of hormone-sensitive lipase and intracellular adipose triglyceride lipase (Lee et al., 2010) and leads to increased glycerol release. Curcumin, a major bioactive compound in turmeric (C. longa) and wild turmeric (C. aromatica), causes inhibition at an early state of adipocyte differentiation (<24 h) by suppressing the dominant transcriptional regulators of adipogenesis, peroxisome proliferator-activated receptor protein-γ (PPAR\(\gamma\)), and CCAAT/enhancer binding protein-α (C/EBP\(\alpha\)) (Ferguson, Nam, & Morrison, 2016). In addition to curcumin, \(\alpha\)-carnitine also inhibits lipid accumulation via stimulation of lipolysis and expression of genes involved in \(\beta\)-oxidation, including hormone-sensitive lipase, carnitine palmitoyltransferase I-a, and \(\alpha\)-acyl-CoA oxidase, which play roles in lipid catabolism (Lee, Lee, & Kim, 2006). Muroski et al. (2007) demonstrated the combined effects of caffeine, soy isoflavones, arginine, and \(\alpha\)-carnitine on triglyceride accumulation, lipolysis, and fatty acid oxidation using 3T3-L1 cells. From these results, we concluded that the decrease in lipid accumulation in 3-, 5-, and 7-day fermented wild turmeric relative to untreated cells may be related to the \(\alpha\)-carnitine and phenolic contents of fermented wild turmeric.

3.5. Inhibitory effects of fermented wild turmeric on nitric oxide production

Inflammation is a biological protection mechanism of the human body against invading pathogens and other danger signals. In this study, the LPS-stimulated RAW264.7 murine macrophage cells were selected for determination of the anti-inflammatory activities of fermented wild turmeric due to their sensitivity to LPS stimulation and release of various inflammatory mediators including nitric oxide (NO), interleukin, and tumor necrosis factor-\(\alpha\) (Lee et al., 2020). First, the cytotoxicity of non-fermented and 1-, 3-, 5-, or 7-day fermented wild turmeric on RAW264.7 murine macrophage cells was measured. The cell viabilities of RAW264.7 murine macrophage cells treated with non-fermented or 1-, 3-, 5-, or 7-day fermented wild turmeric were 93.3 ± 3.6% after 1-day fermentation, 79.2 ± 2.2% after 3-day fermentation, 74.3 ± 0.4% after 5-day fermentation, and returned to the level of non-fermented wild turmeric after 7-day fermentation (104 ± 3.8%) at 50 µg mL\(^{-1}\) (Fig. 3A). At 25 µg mL\(^{-1}\), cell viabilities exceeded 90%; therefore, the nitric oxide assay was performed at 25 µg mL\(^{-1}\). The inhibitory effects of non-fermented and 1-, 3-, 5-, and 7-day fermented wild turmeric were 53.1 ± 3.6%, 56.9 ± 1.3%, 76.7 ± 1.6%, 60.1 ± 2.7%, and 30.1 ± 5.7%, respectively (Fig. 3B). The NO inhibitory effects of 1-, 3-, and 5-day fermented wild turmeric were 1.07-, 1.44-, and 1.13-fold higher than the effect of non-fermented wild turmeric, respectively. However, this effect weakened after 7-day fermentation (Fig. 3B). The highest NO inhibitory activity for wild turmeric was obtained after 3-day fermentation. The increase in NO inhibitory activity was caused by an increase in the phenolic concentration of wild turmeric after fermentation, which led to the suppression of LPS-mediated induction of prostaglandin E2, pro-inflammatory cytokines, and nitric oxide due to reduced levels of cyclooxygenase-2 and nitric oxide synthase in RAW264.7 murine macrophage cells (Kim, Kim et al., 2016). In addition, essential oil from wild turmeric exhibits anti-inflammatory activity via downregulation of the expression of cyclooxygenase-1 and tumor necrosis factor-\(\alpha\) (Xiang et al., 2017).
In the current study, we found that the TPC and TFCC of wild turmeric showed a 1.47- and 2.25-fold increase after 3-day fermentation. Based on UPLC-MS analysis, the wild turmeric showed enhancements of up to 1.17-fold for curcumin, DMC, and BDMC after 1-day fermentation, up to 1.28-fold for ferulic acid after 7-day fermentation, and α-carnitine was newly synthesized; the concentration peaked after 7-day fermentation. In addition, the main volatile compounds in wild turmeric were maintained. The increased concentrations of α-carnitine, curcumin, demethoxycurcumin, bisdemethoxycurcumin, ferulic acid, and phenolic compounds in fermented wild turmeric contributed to the increase in antioxidant activity measured through ORAC and FRAP, anti-lipid accumulation activity in 3T3-L1 cells, inhibition of NO production in LPS-stimulated RAW264.7 murine macrophage cells, and inhibition of melanin formation in B16F10 mouse melanoma cells treated with α-MSH. These findings support the potential application of fermented wild turmeric product in the cosmeceutical and nutraceutical industries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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