Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Higher polyphenol content and bioactivities of freeze-dried *Lycium ruthenicum* fruit and its impact on gut microbiota modulation

Jing Huang[a,1], Xueni Fan[a,1], Xin Li[a], Dingmei Dai[a], Wenjie Huang[b], Dong Ruan[c], Shijuan Yan[b,*], Xiaodan Huang[a,*]

[a] *School of Public Health, Lanzhou University, Lanzhou 730033, China*
[b] *Guangdong Key Laboratory for Crop Germplasm Resources Preservation and Utilization, Agro-biological Gene Research Center, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China*
[c] *Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture and Rural Affairs, State Key Laboratory of Livestock and Poultry Breeding, Guangdong Key Laboratory of Animal Breeding and Nutrition, Guangzhou 510640, China*

ARTICLE INFO

Article history:
Received 6 October 2023
Received in revised form 14 December 2023
Accepted 3 April 2024

Keywords:
Lycium ruthenicum
Drying methods
Antioxidant activity
Anti-inflammatory activity
Gut microbiota
Short chain fatty acids

ABSTRACT

Black wolfberry (*Lycium ruthenicum*) is enriched in phytochemical metabolites which can benefit human health. However, few studies have examined the effects of different fruit drying methods on its polyphenol content, antioxidant activity, and anti-inflammatory activity. In addition, whether and how consuming dried black wolfberry affects gut microbiota has not been reported. This study assessed the phytochemical profile and bioactivities of black wolfberry dried through different methods, and subsequently characterized changes in human fecal microbiota associated with freeze-dried black wolfberry *in vitro*. The results showed that freeze-dried samples retained higher total phenolics ((49.68 ± 1.62) mg GAE/g DM), tannins ((38.64 ± 1.35) mg GAE/g DM), and proanthocyanidins ((3.35 ± 0.30) mg/g DM) compared to sun drying or hot air drying ($P < 0.05$), and exhibited higher antioxidant and anti-inflammatory activities. In human fecal inoculum bioreactor fermentations, freeze-dried black wolfberry was associated with increased species richness and α-diversity. At the genus level, fermentations treated with black wolfberry had a higher abundance of lactic acid bacteria including *Lactococcus*, *Bifidobacterium*, *Lactobacillus*, *Pediococcus*, and *Weissella*, as well as butyrate-producing bacteria compared to the untreated samples, suggesting enrichment for taxa associated with a healthy gut microbiome. In addition, the black wolfberry treatment group had higher levels of short-chain fatty acids, which were consistent with PICRUSt2 inference. This study defines an optimal method for black wolfberry preservation to retain the beneficial compounds, and provides a foundation for further exploration of its potential benefits for human gut microbiota.

1. Introduction

Black wolfberry (*Lycium ruthenicum*) is an important economic plant resource, which is widely distributed across arid desert and saline-alkali areas[1]. As an edible fruit often consumed in western China and South Asia, it contains various bioactive compounds with numerous health benefits. Black wolfberry has been recorded in Tibetan medical history as a traditional herb for the treatment of abnormal eye disease, menopause, menstruation, and hypertension[2]. In addition, the extract of black wolfberry, known for its bioactive properties and potential cognition-improving effects, has been suggested in studies to serve as a mitochondrial-protective agent for the

[1] These authors contributed equally to this work.
[*] Corresponding authors.
E-mail address: huangxiaodan@lzu.edu.cn (X.D. Huang); shijuan@agrogene.ac.cn (S.J. Yan)
Peer review under responsibility of Beijing Academy of Food Sciences.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2024.9250233

treatment of neurodegenerative diseases such as Alzheimer's[3]. There are various constituents in black wolfberry, including proteins, dietary fibers, soluble sugars, vitamins, minerals, amino acids, ascorbic acid, flavonoids, and polysaccharides[4-5]. Due to this wide variety of compounds with purported benefits and potential therapeutic value for human health, black wolfberry has aroused considerable research attention. In particular, black wolfberry contains an especially large number and high concentration of polyphenols, along with polysaccharides, which are likely responsible for its antioxidant activity[3-4]. A comparison research between red goji berry and black wolfberry showed that black wolfberry had higher contents of phenolic, condensed tannin, and monomeric anthocyanin, and also exhibited higher antioxidant activity[6].

Fresh fruit of black wolfberry has high water content and is prone to mildew, leading to the necessity of post-harvest preservation for long-term storage, especially through drying to retain the bioactive compounds. However, different drying methods are associated with different effects on the compounds retained in the fruit and production costs related to drying temperature[7]. Sun drying (SD), hot-air drying (HD), and freeze drying (FD) are all commonly used methods for post-harvest processing of black wolfberry, and all of them reportedly influence the physical properties, bioactive compounds, and antioxidant activity of fruit, and consequently the bioaccessibility and function of its metabolites[8-10]. Therefore, it is necessary to evaluate the effects of various drying methods to determine the optimal method for retaining the highest quantity of potentially beneficial compounds. FD is currently considered one of the best drying methods to preserve food quality, and it has been shown to retain greater antioxidant capacity and higher contents of anthocyanins and phenolics compared to other methods[11].

Polyphenols are classified by their structural features into phenolic acids (such as benzoic acid and cinnamic acid derivatives), flavonoids, and non-flavonoids[12], which are secondary metabolites with a wide range of physiological and biological activities[4,13]. The International Scientific Association for Probiotics and Prebiotics has expanded the activities of polyphenols to include prebiotics[14], which are reported to exhibit functions including enhancing immunity, anti-oxidation, anti-inflammation, anti-tumor, hepatic protection, anti-fatigue and hypoglycemic effects[3,15]. Recently, studies have shown the important roles of polyphenols in black wolfberry in protecting the integrity of the intestinal barrier and reducing intestinal inflammation by plant metabolites[16]. The gut microbiota are considered a hidden organ in humans and are involved in nutrient acquisition and utilization of various metabolic pathways, thus supporting the host immune system[17]. As important prebiotic metabolites, polyphenols can regulate gut microbiota and exert anti-inflammatory and immunomodulatory effects[18]. A systematic review by Cardona et al.[19] explored the benefits of polyphenolic compounds on gut microbiota and human health and proposed a "two-way relationship" between polyphenols and microbiota. Other than a source of polyphenols, the bioaccessibility of polyphenols also leads to various functions related to absorption in the gastrointestinal tract[20]. Generally, complex polyphenols can be decomposed into small molecules and subsequently absorbed. At the same time, undegraded parts enter the gastrointestinal tract and support the microbial ecosystem by providing different substrates to alter gut metabolic functions, resulting in 'prebiotic-like' effects[18,21]. Plant hosts highly rely upon these bioactive compounds and their functions. Other studies have shown that black wolfberry supplements not only provide a potential source of bioactive compounds but also might provide a new strategy for preventing and ameliorating inflammatory bowel disease[22]. However, the influence of black wolfberry consumption on gut microbiota has yet to be investigated.

In our investigation to ascertain the preservation method for black wolfberry, we conducted a comprehensive comparison of various drying techniques, with a particular focus on their impacts on the phytochemical composition of black wolfberry, especially its phenolic profile, as well as its antioxidant and anti-inflammatory activities. In addition, black wolfberry dried by the technique with higher bioactivities was used to further evaluate the effects on gut microbiota and microbial short chain fatty acids (SCFAs) production *in vitro*. This study reveals the effects of different drying methods on phenolic metabolites, and the associated antioxidant and anti-inflammatory effects of black wolfberry. This work also extends knowledge of the role of black wolfberry in enriching functional beneficial gut microbiota.

2. Materials and methods

2.1 Plant materials

Black wolfberry was collected from Ningxia, China (altitude 1 200 m, latitude 35° 14′–39° 23′N, longitude 104° 17′–107° 39′E). Mature fruits 10 days after the breaker stage were sampled and transferred to the laboratory on dry ice. Before further testing, black wolfberry fruits were subject to a selection of uniform size (mean diameter 8–15 mm), removing small and unripe berries, as well as other impurities, such as stems, leaves, and petioles.

2.2 Drying conditions

Fresh black wolfberry fruits were dried by various drying methods: in SD group, black wolfberry fruits were laid on a flat surface in an open area, and sun-dried for 12 h every day (08:30–20:30) for 5 days. FD was performed in a pilot-scale freeze drier (LABCONBO, Missouri, USA) with 0.125 Torr at 55 ℃, the total processing time was 72 h. Hot-air drying procedure was conducted in a hot-air oven (GERIRM, Cologne, Germany) at 60 ℃ (HD60) and 50 ℃ (HD50) lasting for 18 h, respectively. Variable temperature drying (VD) was performed at variable temperatures in the hot-air oven which was set at 40 ℃ for 6 h, 50 ℃ for 6 h, and 60 ℃ for 6 h, respectively. After drying, the samples were reweighed, and the initial moisture was measured by setting the hot-air drying at 100 ℃ overnight for dry matter-based (DM) calculation and were then ground into a fine powder (passed through a 40-mesh sieve) and stored at −80 ℃ until required for further investigation.

2.3 Sample extraction

A total of 2.0 g black wolfberry powder was extracted with 30 mL of 80% methanol in an ultrasonic bath (40 kHz) for 30 min. The extract was then centrifuged at 10 000 × *g* for 10 min, the supernatant was re-extracted with 10 mL 80% methanol and the step was repeated twice and then subjected to analysis.

2.4 Physicochemical properties and phytochemical compounds analysis

2.4.1 Physicochemical properties

The solubility and bulk density of black wolfberry obtained from different drying processes were measured according to the previous study[23]. All samples were measured 3 times.

2.4.2 Polysaccharide content

Polysaccharide content determination was carried out according to the phenol-sulfuric acid method based on the colorimetric reaction of monosaccharides with phenol in the presence of concentrated sulfuric acid with slight modification[4]. Briefly, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the glucose standard solution (100 mg glucose/L) were added and filled with distilled water to 1 mL. Subsequently, 1 mL phenol was added to the standard working solution and 5.0 mL sulfuric acid was quickly added, and allowed to incubate at room temperature for 10 min. The mixture was thoroughly mixed and incubated at 30 ℃ for 20 min, and then the absorbance was measured at 490 nm. The results were expressed as mg/g DM.

2.5 Polyphenols determination

Total polyphenols content and tannin content were measured according to the Folin-Ciocalteu/ polyvinylpolypyrrolidone (PVPP) assay by Palacios et al.[24] with slight modification. In brief, the extract was dissolved in methanol to make a solution of 1.0 mg/mL. Then, 1.0 mL of each sample solution and 0.5 mL of 0.5 mol/L Folin-Ciocalteu reagent (Sigma-Aldrich, Shanghai, China) were accurately added to a volumetric flask, and the reaction lasting for 1 min, then neutralized with 2.5 mL saturated sodium carbonate. The solutions were incubated at 25 ℃ for 40 min, and the absorbance was measured at 725 nm with a UV-5300PC spectrophotometer (Metash Instrument Co., Ltd, Shanghai, China). Gallic acid equivalents in the range 0.002–0.150 mg/mL were used for construction of the calibration curve. Tannin content was calculated as the difference between total phenols and phenols remnant after precipitation with PVPP, the results were expressed as mg/g DM of gallic acid equivalents (GAE). Total flavonoids content was measured according to the previously described method from Liu et al.[4], results were expressed as mg/g DM rutin equivalents (RE). Proanthocyanidins content was determined by the butanol-HCl method[25], results were expressed as mg/g DM.

2.6 Liquid chromatography-mass spectrometer (LC-MS) quantification of phenolic components

Polyphenol quantification was performed as previously described[26]. Briefly, 50 μg dried aliquot was re-suspended in 150 μL UPLC-grade methanol: water (1:1, *V/V*), then 5 μL was taken for chromatographic detection (XBridge BEH C_{18} column, 2.1 mm × 100 mm, 2.5 μm; Waters), with 0.1 mL/min flow rate at 40 ℃. The extracts were separated and eluted, and then the eluates were used for MS analysis and polyphenols quantification. MS data were recorded for 35 min and analysed using Analyst software version 1.5.2 (AB SCIEX, Foster City, CA, USA).

2.7 In vitro bioaccessibility, antioxidant activity and anti-inflammatory activity assays

2.7.1 Bioaccessibility

The bioaccessibility of bioactive compounds was determined according to Vitali et al.[27] with minor modifications. In brief, 1.5 mL pepsin (20 g/L in 0.1 mol/L HCl) was added to 30 mL of distilled water and the mixture was mixed with 1.5 g of sample powders (pH 2.0). Then, the mixture was incubated at 37 ℃ for 1 h in a shaking water bath (Julabo, Seelbach, Germany). At the end of the incubation time, the pH was adjusted to 7.2 using 1 mol/L $NaHCO_3$ to terminate gastric digestion. Then, 7.5 mL of pile/pancreatin solution (12 g bile salt and 2 g pancreatin in 0.1 mol/L $NaHCO_3$) and 7.5 mL NaCl/KCl (120 mmol/L NaCl and 5 mmol/L KCl) were added to the mixture. The environment simulated intestinal digestion for a period of 2.5 h at 37 ℃ in a shaking water bath. The final mixture was centrifuged at 5 000 × *g* for 10 min and the supernatant was subjected to determination of bioaccessible phenolics using the Folin-Ciocalteu method and expressed as gallic acid equivalents (mg of GAE/g DM). The bioaccessibility of phenolic compounds was calculated as the percentage of bioavailable phenolic compounds in the total phenolic compounds of black wolfberry.

2.7.2 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay

The antioxidant activity was evaluated *in vitro* by DPPH free radical scavenging assay according to Yang et al.[28] with slight modification. For the DPPH assay, 0.15 mL sample was added to DPPH solution (0.5 mmol/L). The mixture was shaken and reacted 30 min at 37 ℃ in dark. Then, the absorbance was read at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$I(\%) = \left(1 - \frac{(Ai - Aj)}{Ac}\right) \times 100 \quad (1)$$

Where *I* is DPPH free radical scavenging activity; *Ac* is the absorbance of DPPH solution without sample; *Ai* is the absorbance of the test sample mixed with DPPH solution; *Aj* is the absorbance of the sample without DPPH solution.

2.7.3 Oxygen radical absorbance capacity (ORAC) assay

ORAC assays were carried out according to Liu et al.[4]. Phosphate buffer was prepared at 37 ℃ (pH 7.4), the peroxyl radicals were produced by 2,2′-azobis (2-amidino-propane) dihydrochloride. Fluorescence conditions were set with excitation at 485 nm and emission at 520 nm. Trolox (0–50 μmol/L) was used for the standard curve. Results were expressed in μmol TE/g DM.

2.7.4 Ferric reducing/antioxidant power (FRAP)

The FRAP assay was performed according to the instruction manual of the kit purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Weigh and dilute an appropriate 100 mmol/L $FeSO_4 \cdot 7H_2O$ solution to 0.15–1.50 mmol/L. A standard solution was prepared using distilled water or a sample preparation solution. Results were expressed as mmol Fe^{2+}/g DM.

2.7.5 Anti-inflammatory assays

The anti-inflammatory effect of black wolfberry extracts was determined according to a method used in the previous study[26] as modified from Torres-Rodriguez et al.[29]. In 96-well plates, RAW 264.7 cells were seeded at a density of 1×10^3–4×10^3 cells/well and incubated in Dulbecco's Modified Eagle Medium for 24 h at 37 ℃ with 5% CO_2. Then the cells were treated with various extracts from 5 black wolfberry samples and lipopolysaccharide (LPS) (1 μg/mL) in the same plates. Nitrite levels in culture supernatants were measured using Griess reagent containing 1% (*m*/*V*) sulfanilamide and 0.1% (*m*/*V*) naphthyl ethylenediamine dihydrochloride in 5% phosphoric acid. After 10 min of incubation, the nitrite concentration was obtained at 540 nm with a microplate reader (Bio-Rad Laboratories, CA, USA). The nitric oxide (NO) inhibition rate was calculated as the percentage relative to the control, with the formula:

$$\text{NO inhibition rate (\%)} = 1 - \frac{\text{NO production (sample)}}{\text{NO production (LPS)}} \times 100 \quad (2)$$

Results were expressed as the anti-inflammatory concentration that reduced the NO inhibition rate by 50% (IC_{50}, μg/mL). The concentrations of interleukin (IL)-1β and IL-6 were quantified in the supernatants of the culture medium using commercially available ELISA kits (Solarbio, Beijing, China). The analyses were conducted in accordance with the manufacturer's protocols.

Anti-inflammation activities were assessed using the colorimetric protease inhibitory method as per the protocol outlined by Islam et al.[30]. After extraction and incubation, the resulting solution underwent centrifugation at 5 000 r/min for 10 min, and the absorbance was measured using a UV-spectrophotometer at 280 nm against a blank.

2.8 In vitro intestinal fermentation model establishment

2.8.1 Fecal inoculums

The fecal inoculums were prepared according to the method from Zhao et al.[31]. Fresh feces were collected from 4 healthy donors (2 females and 2 males, 26 years old), who had not been treated with antibiotics for at least 3 months. All procedures were approved by the Medical Ethics Committee of the School of Public Health (IRB21022608). Informed consent forms were signed by all participants before sampling. In order to obtain 10% (*m*/*V*) fecal suspension, 100 μL of pooled fecal slurry were subjected to homogenization with sterilized phosphate-buffered saline (pH 7.4). After filtration through 4 layers of cheesecloth, fecal inoculums were collected until further operation within 1 h.

2.8.2 In vitro intestinal fermentation

Basal nutrient medium (1 L) was prepared by adding 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 40 mg K_2HPO_4, 40 mg KH_2PO_4, 10 mg $MgSO_4$, 10 mg $CaCl_2$, 2 g $NaHCO_3$, 0.5 g cysteine-HCl, 0.5 g bile salt, 2 mL Tween-80, 5 mL vitamin K_1, and 20 mg hemin (dissolved by the addition of 6 mol/L NaOH) into distilled water (pH 7.4). FD samples were diluted with 1× phosphate-buffered saline (PBS) at pH 7.0. The study applied 3 treatments with different amounts of FD black wolfberry as NAG (0 g/50 mL PBS, no addition group), LAG (3 g/50 mL PBS, low addition group), and HAG (6 g/50 mL PBS, high addition group). A total of 1 mL of the respective fecal sample was added to the 9 mL basal nutrient medium. All fermentation experiments were conducted in triplicate in an anaerobic jar and incubated at 37 ℃ in a shaking incubator for 0, 4 and 24 h, respectively.

2.9 Microbial and SCFAs analysis

2.9.1 Polymerase chain reaction (PCR) amplification and high-throughput sequencing

Fermentation samples of 3 treatments at 0, 4 and 24 h were collected and genomic DNA was extracted using the TIANamp stool DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). DNA yield and purity were determined by a DNA spectrophotometer (ND-1000; NanoDrop, DE, United States). Normalized genomic DNA was applied to amplify the V3–V4 hypervariable region of the 16S rRNA gene (barcode PCR) with a set of primers (F: 5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer (R: 5′-GACTACHVGGGTATCTAATCC-3′). A total of 25 μL reaction volume with Phusion™ High-Fidelity PCR Master Mix (New England Biolabs) was used in the PCR reactions with cycling parameters of 98 ℃, 1 min; 30 cycles of 98 ℃ for 10 s, 55 ℃ for 30 s, 72 ℃ for 30 s; final extension at 72 ℃ for 5 min. The final PCR products were separated and visualized by agarose gel electrophoresis and then subjected to sequencing on an Illumina NovaSeq platform (Illumina, CA, USA).

Samples are identified by a paired-ended barcode reading. Paired-end reads were merged with tags by using FLASH (v1.2.11)[32] after deleting the sequences of barcodes and primers and the < 100 bp reads *via* TrimGalore, and then sequences are clustered by UPARSE into operational taxonomic units (OTUs) with 97% similarity cut-off after removing the noise and chimera using the gold. fa database (http://drive5. com/uchime/gold. fa). OTUs were thus classified based on Ribosomal Database Projet (RDP) Release 9 201203 by Mothur. Rarefaction and α-diversity were analyzed by Mothur. Principal coordinates and visual differences of multi-dimensional data from fermentation broth samples were analyzed by principal coordinate analysis (PCoA). The bacterial function prediction was carried out using PICRUSt2 (Version 2.1.2-b)[33]. The 16S rRNA data can be accessed at the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA890012).

2.9.2 Detection of SCFAs

The thawed fermented broths was subjected to centrifugation at 3 850 × *g* for 15 min at 4 ℃. After this, 1 mL of the supernatant was mixed with 0.2 mL of metaphosphoric acid solution, which contained 2-ethylbutyric acid as an internal standard. The mixture was left to stand for 30 min at 4 ℃. Afterward, the solution was again centrifuged at 3 850 × *g* for 15 min at 4 ℃. The supernatant was then filtered using a 0.22 μm filter paper and used in gas chromatography (SP-3420A; Beijing Beifen-Ruili Analytical Instrument Co., China) under the following conditions: 90 ℃ for 1 min, followed by increasing to 120 ℃ at 10 ℃/min for 1 min, from 120 to 150 ℃ at 10 ℃/min, and held at 150 ℃ for 3 min. Both the spray hole temperature and the auxiliary chamber temperature were 250 ℃[34].

2.10 Statistical analysis

Determinations of the investigated indices were carried out in 3 replicates and the results were expressed as mean ± standard deviation (SD),

all the component content and antioxidant value were expressed as DM, and were analysed using SPSS 20.0 and GraphPad Prism 8.0.2 software. Differences between means were analyzed firstly by analysis of variance (ANOVA), the association between various bioactive compounds through Spearman's correlation analysis. Non-parametric factorial Kruskal-Wallis sum-rank tests were performed to test for differences among groups at the bacterial phylum and genus levels, and Dunn's test was performed to separate means where significance was found. Tukey-adjusted P values were used to separate means and statistical significance was accepted at $P < 0.05$.

3. Results

3.1 FD retains more phytochemicals and enhances solubility compared to other conditions

Physicochemical properties and phytochemical compounds of black wolfberry dried under different conditions are presented in Fig. 1. Evaluation of powdered black wolfberry solubility, an indicator of behavior in aqueous solution, showed that its solubility was affected by drying conditions, which ranged from 48.52% to 59.25%, in the order VD < HD50 < HD60 < SD < FD from least to greatest solubility of treatments. The loose and packed bulk density index was lowest in FD samples (0.47 g/mL) compared with other drying methods ($P < 0.05$), while SD sample had the highest bulk density (0.56 g/mL, $P < 0.05$).

Characterization of black wolfberry phytochemical compound profiles revealed that drying conditions affected total polyphenols, polysaccharides, total flavonoids, tannins and proanthocyanidins (Fig. 1). Polysaccharide contents were highest in SD samples, and lowest in FD (30.73 mg/g DM *vs*. 20.88 mg/g DM; $P < 0.05$), while total polyphenols content was highest in FD samples (49.68 mg GAE/g DM; $P < 0.05$) and lowest in the VD samples (30.29 mg GAE/g DM; $P < 0.05$). Tannin and proanthocyanidins contents shared the same trend as total polyphenols, in which the highest levels of these compound groups were detected in FD and the lowest levels were found in VD samples (tannin: 38.64 mg GAE/g DM *vs*. 20.68 mg GAE/g DM; proanthocyanidins: 3.35 mg/g DM *vs*. 1.10 mg/g DM, respectively; $P < 0.05$). These results illustrated that the FD treatment resulted in higher retention of total polyphenols, tannins, and proanthocyanidins than other drying conditions.

3.2 More specific phenolics detected by LC-MS and higher bioaccessibility of black wolfberry under HD60 condition

Quantification of specific phenolics in black wolfberry fruits, subjected to diverse drying conditions, was conducted using LC-MS, revealing the identification of a total of 28 phenolic cations and anions (Figs. 2 and 3). Notably, quercetin-3-*O*-rutinose (1 213.56–1 982.08 μg/g DM) emerged as the predominant compound, demonstrating the highest abundance among the detected phenolics. Subsequently, compounds such as naringin (820.77–1 998.04 μg/g DM), rutin (1 265.36–1 599.36 μg/g DM), and narcissoside (939.23–1 667.67 μg/g DM) were also identified. The contents of phenolic substances showed considerable variation among drying methods, with 6 phenolics, including quercetin, rutin, kaempferol-3-*O*-rutinoside, narcissoside, salicylic acid and procyanidin A1, found at their lowest levels in FD samples, while naringenin chalcone was at its highest levels compared with other drying treatments ($P < 0.05$). By contrast, 2-methylbenzoic acid, kaempferol-3-glucuronide, ferulic acid, quercetin-3-*O*-rutinose, luteolin, and kaempferol were the most abundant in SD conditions. In addition, coumarin, taxifolin, quercetin, 4*H*-1-benzopyran-4-one, pyrogallol and isoquercitin had the highest concentrations under VD conditions ($P < 0.05$).

According to *in vitro* gastrointestinal digestion assays, the bioaccessibility of phenolic compounds in black wolfberry ranged from 32.06% to 40.02% (Fig. 4A). The assessment measures the quantity of phenolics released from the food matrix for intestinal absorption after digestion. The drying conditions of HD60 and VD, both processed at a high temperature of 60 °C, resulted in higher bioaccessibility compared to SD, FD, or HD50 ($P < 0.05$). In summary, our investigation revealed a higher detection of phenolic substances through LC-MS analysis under hot air drying conditions, notably emphasizing the pronounced impact observed at 60 °C drying conditions, and the bioaccessibility of hot-air drying conditions of 60 °C was higher.

3.3 FD black wolfberry samples have higher antioxidant and anti-inflammatory capacity which is correlated with polyphenols

To assess whether drying conditions affected black wolfberry antioxidant activity, DPPH, FRAP and ORAC assays were used to compare free radical scavenging and antioxidant properties of

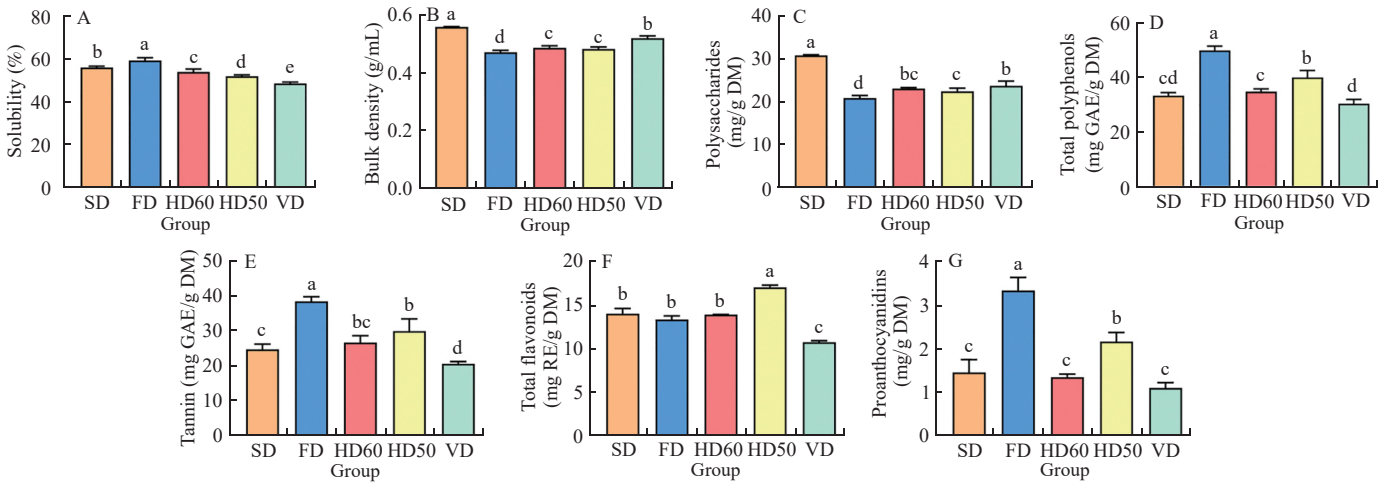

Fig. 1 Physicochemical properties and phytochemical compounds of black wolfberry. (A) Solubility; (B) Bulk density; (C) Polysaccharides; (D) Total polyphenols; (E) Tannin; (F) Total flavonoids; (G) Proanthocyanidins. Different lowercase letters indicate significantly different at $P < 0.05$.

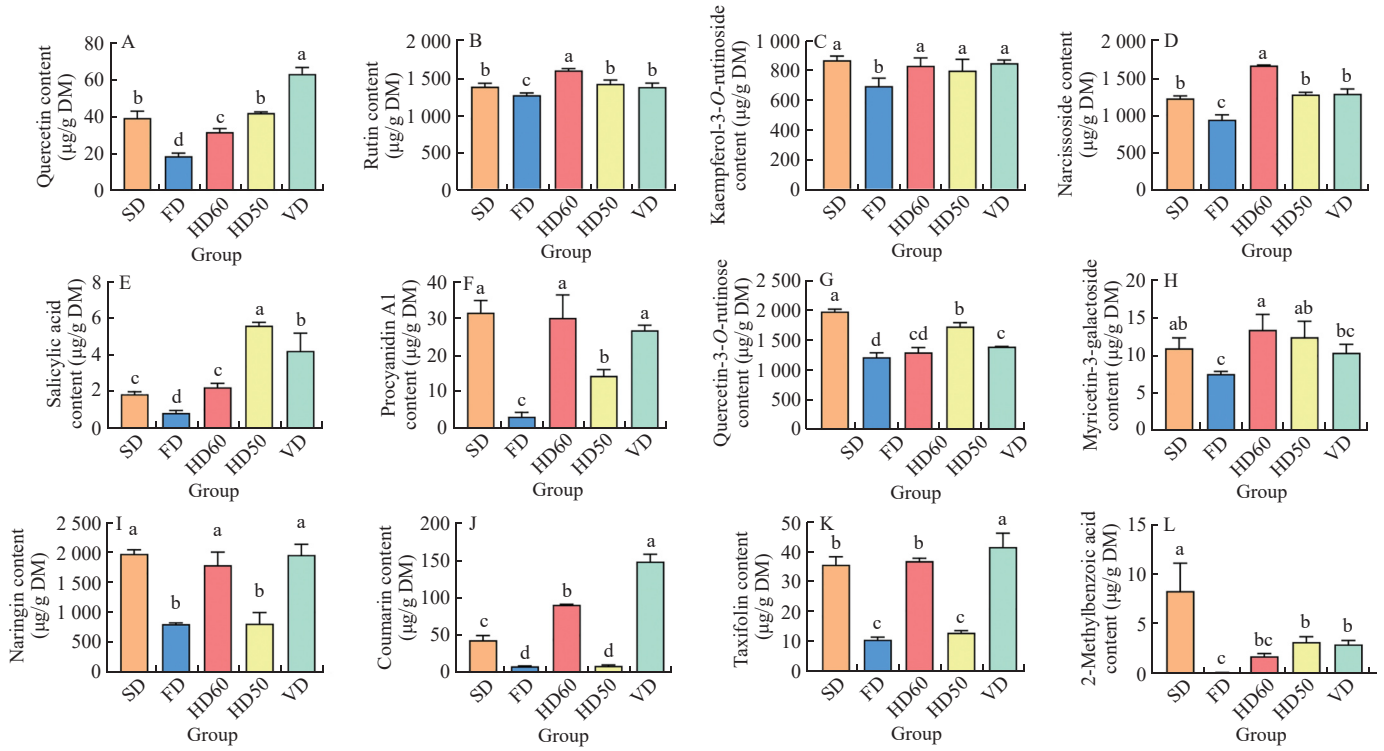

Fig. 2 The content of phenolic compounds of black wolfberry in FD is lower. (A) Quercetin; (B) Rutin; (C) Kaempferol-3-*O*-rutinoside; (D) Narcissoside; (E) Salicylic acid; (F) Procyanidin A1; (G) Quercetin-3-*O*-rutinose; (H) Myricetin-3-galactoside; (I) Naringin; (J) Coumarin; (K) Taxifolin; (L) 2-Methylbenzoic acid. Different lowercase letters indicate significantly different at $P < 0.05$.

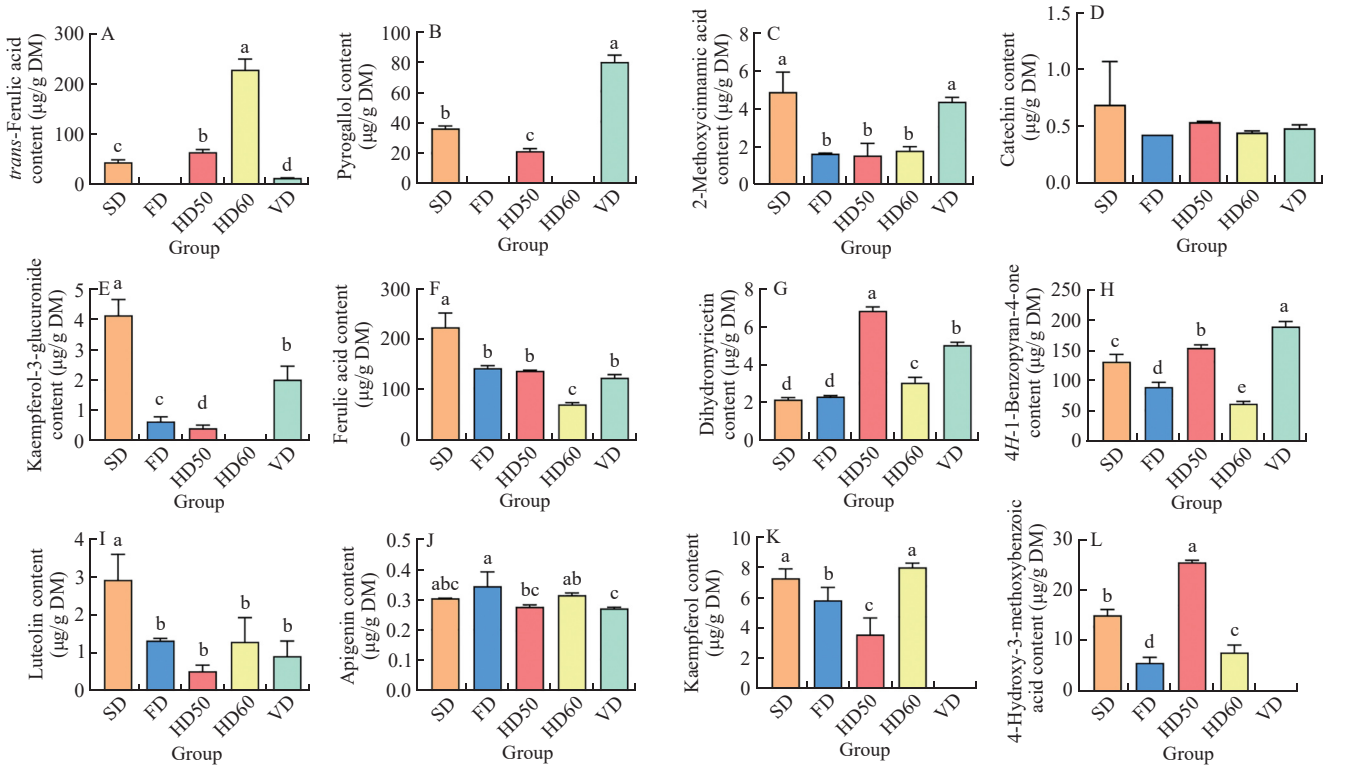

Fig. 3 The content of phenolic compounds of black wolfberry in different drying methods. (A) *trans*-Ferulic acid; (B) Pyrogallol; (C) 2-Methoxycinnamic acid; (D) Catechin; (E) Kaempferol-3-Glucuronide; (F) Ferulic acid; (G) Dihydromyricetin; (H) 4*H*-1-Benzopyran-4-one; (I) Luteolin; (J) Apigenin; (K) Kaempferol; (L) 4-Hydroxy-3-methoxybenzoic acid; (M) Typhaneoside; (N) Isoquercitrin; (O) Sinapic acid; (P) Naringenin chalcone. Different lowercase letters indicate significantly different at $P < 0.05$.

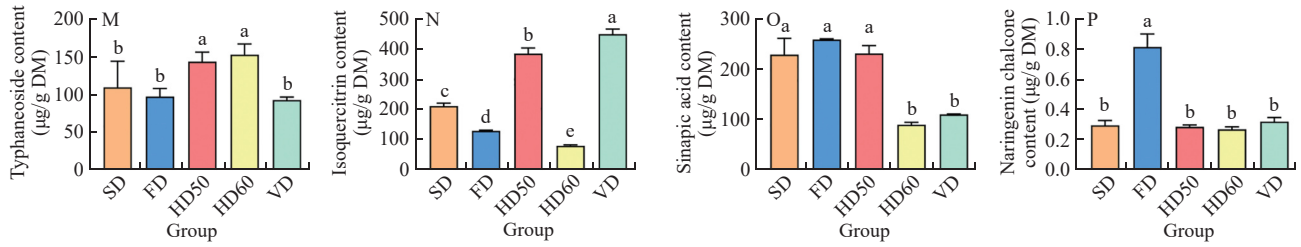

Fig. 3 (*Continued*)

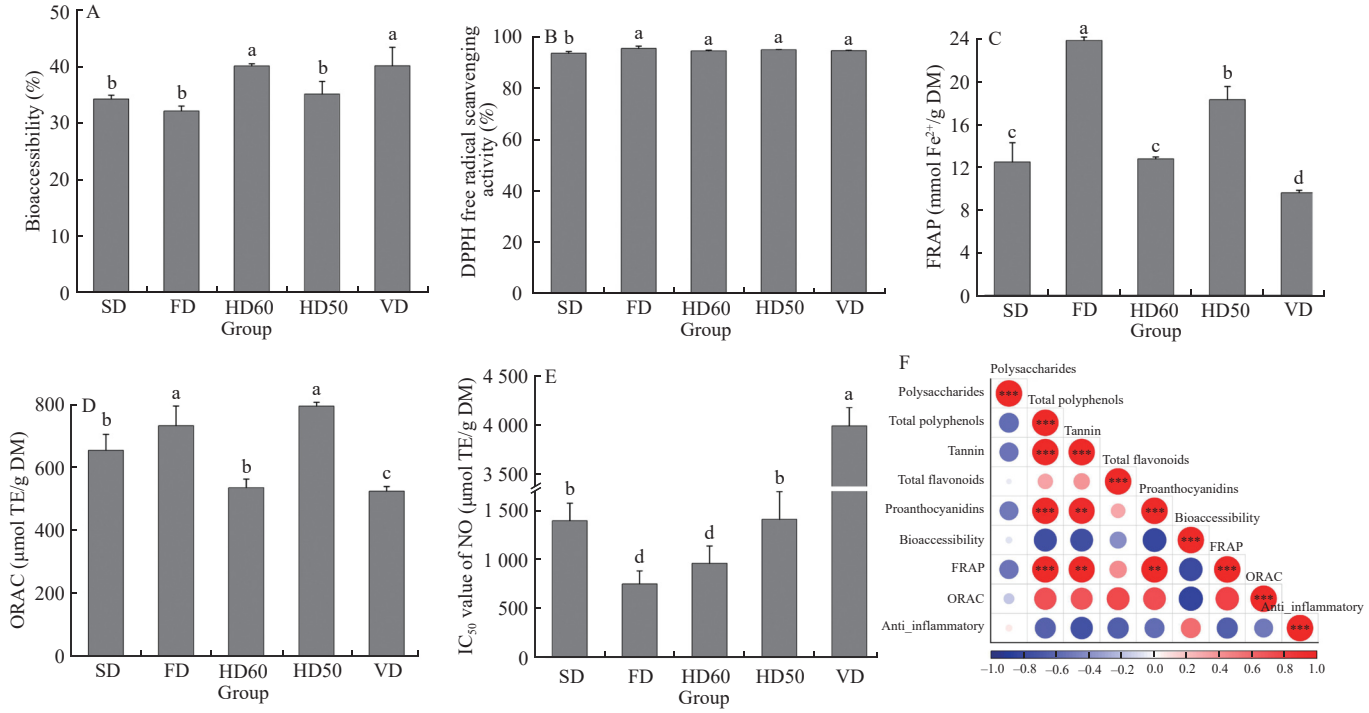

Fig. 4 Bioaccessibility, antioxidant and anti-inflammatory activity of extracts from black wolfberry with various drying conditions. (A) Bioaccessibility of black wolfberry under different drying conditions. (B) DPPH free radical scavenging activity of black wolfberry fruits; (C) FRAP of black wolfberry fruits; (D) ORAC of black wolfberry fruits; (E) Effect of black wolfberry fruits on NO production in priming RAW264.7 cell with LPS. Error bars show standard deviation. Different lowercase letters indicate significant difference ($P < 0.05$, ANOVA). (F) The correlation between phytochemical in black wolfberry and their bioactivities. Positive correlations are represented by red color and negative correlations by blue color. Color intensity are proportional to the correlation coefficients (Spearman correlation). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

samples prepared by each drying treatment (Figs. 4B-D). DPPH free radical scavenging assays indicated that activity varied from 93.49% to 95.37% among treatments. SD extracts had the lowest antioxidant capacity (93.49% of DPPH inhibition; $P < 0.05$), while no significant differences were detected between the other groups. FRAP measurements of antioxidant activity indicated that FD had the highest FRAP values ((23.81 ± 0.31) mmol Fe^{2+}/g DM; $P < 0.05$), followed by HD50 ((18.27 ± 1.24) mmol Fe^{2+}/g DM; $P < 0.05$), while VD samples had the lowest antioxidant capacity ((9.58 ± 0.24) mmol Fe^{2+}/g DM; $P < 0.05$) in FRAP assays. ORAC were similar to FRAP, ranging from 525.68 to 796.07 μmol TE/g DM, with the highest antioxidant activity detected in FD and HD50 samples ((734.00 ± 62.71) and (796.07 ± 12.02) μmol TE/g DM, respectively), and the lowest activity was observed in VD samples ((525.68 ± 15.03) μmol TE/g DM; $P < 0.05$). These results collectively showed that FD processing resulted in fruit retaining higher antioxidant properties than other drying methods.

The assessment of black wolfberry's anti-inflammatory activity through NO production assays was depicted in Fig. 4E and validated suppressive effect of IL-6 and IL-1β. FD samples exhibited significantly lower IC_{50} values ((756.11 ± 132.89) μg/mL DM; $P < 0.05$), indicative of a higher anti-inflammatory capacity compared to VD ((3 992.66 ± 188.68) μg/mL DM), SD ((1 399.97 ± 179.33) μg/mL DM) and HD50 ((1 414.59 ± 283.20) μg/mL DM), followed by HD60 ((964.93 ± 177.10) μg/mL DM); and IC_{50} value for HD60 group was slightly higher than of FD ($P > 0.05$). VD samples had the highest IC_{50} values, suggesting that this drying method resulted in a greater loss of anti-inflammatory capacity than other treatments. Colorimetric protease inhibitory method revealed that the inhibitory rate of all extracts at various concentrations was consistently below 4%, and there were no differences in black wolfberry samples between different drying conditions (Table S1).

Correlation analysis was conducted between phytochemical concentrations and bioactivity for black wolfberry samples from different drying treatments to identify compounds potentially relevant to antioxidant activity (Fig. 4F). There is a significant positive correlation between total polyphenols, tannins, and proanthocyanins with FRAP

(r = 0.98–0.99; P < 0.01). Among the phytochemical compounds, tannin contents had a positive correlation with total polyphenols and proanthocyanins (r = 0.99 and 0.97; P < 0.05), while total polyphenols were highly correlated with proanthocyanins (r = 0.99; P < 0.05). In conclusion, FD black wolfberry samples exhibited superior antioxidant and anti-inflammatory capabilities, showcasing a positive correlation with the polyphenols. These findings underscored the superior anti-inflammatory potential of FD, positioning it as a favourable method for preserving and enhancing the bioactive properties of black wolfberry.

3.4 FD black wolfberry enrich for potentially beneficial taxa and SCFAs production in human gut microbiota in vitro

Considering the potentially heightened bioactivity observed in FD samples, FD black wolfberry was chosen as the substrate for testing microbial enrichment in an *in vitro* bioreactor designed to simulate the human gastrointestinal tract. Basal nutrient medium was supplemented with no FD black wolfberry (NAG), 3 g FD black wolfberry (LAG), or 6 g FD black wolfberry (HAG) and 16S rRNA gene sequencing was used to characterize the gut microbiota pooled from 2 male and 2 female healthy volunteers. A total of 924 OTUs were identified, with all 9 groups (n = 3 time points per group) sharing 78 OTUs (8.44%) in common (Fig. 5). In each treatment group, distinctive OTUs were identified across various time points. Specifically, for the NAG treatment, 75, 66, and 66 unique OTUs were observed in 0, 4 and 24 h samples, respectively; for LAG group, 49, 60, and 87 unique OTUs were detected in the corresponding time points; And 81, 38, and 78 unique OTUs were present in HAG 0, 4, and 24 h samples, respectively. These findings highlighted the dynamic and treatment-specific shifts in the microbial community composition over different time intervals.

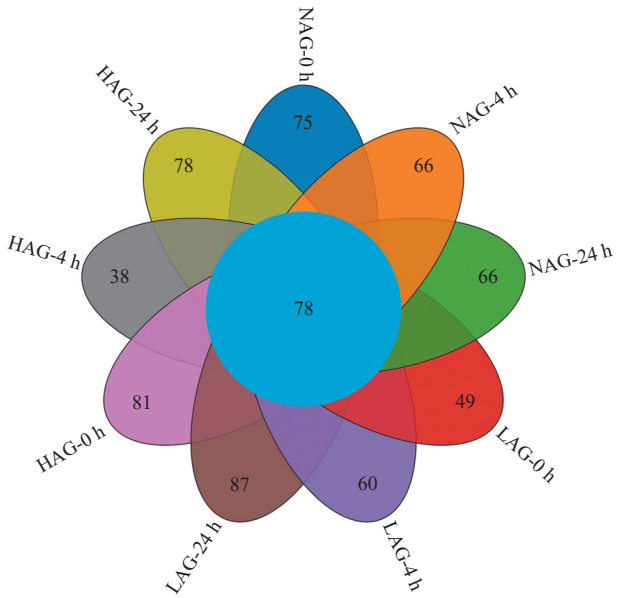

Fig. 5 Unique and common OTU Venn diagrams between groups 0, 4, and 24 h.

Analysis of microbial α-diversity by Observed species richness and Chao1 indexes revealed a decrease in the first 4 h (Fig. 6A; P < 0.001), which was restored by 24 h of fermentation in the LAG and HAG groups. Nevertheless, in NAG group, species richness exhibited a consistent decline over the 24-h fermentation period. Evaluation of Shannon index provided additional support for this trend, indicating a reduction in microbial diversity within the initial 4 h. However, by the end of the 24-h incubation period, a slight rebound in microbial diversity was observed in both NAG and HAG groups. In contrast, the LAG fermentations showed a distinct pattern, with microbial diversity progressively enriching from 0 to 24 h. These results underscored the dynamic nature of microbial communities, with fermentation time and substrate-specific effects influencing the observed changes in species richness and diversity.

To better understand the effects of FD black wolfberry on β-diversity of gut microbiota, we conducted principal coordinate analysis (PCoA) based on weighted UniFrac distance. This analysis indicated that microbiota sampled at 24 h of fermentation showed significant separation between NAG, LAG and HAG groups (Fig. 6B), with the principle PC1 axis explaining approximately 83.82% of the observed variation between samples. Permutational multivariate analysis of variance (PERMANOVA) (Fig. 6C) analysis further supported the significant difference in β-diversity among groups (P = 0.003 1).

Phylum level analysis of bacterial composition in the different groups during fermentation (Fig. 7) showed that Bacteroidetes were the most prevalent phylum (28.72%–43.12%), followed by Firmicutes (12.02%–41.36%), and Proteobacteria (7.9%–46.41%). Differences among the NAG, LAG, and HAG bacterial communities after 24 h of fermentation corresponded with the abundance of Fusobacteria (Fig. 8A), which decreased concomitantly with the addition of black wolfberry extracts (NAG > LAG > HAG; P < 0.05). By contrast, Firmicutes and Actinobacteria increased in abundance with the addition of higher amounts of black wolfberry (NAG < LAG < HAG; P < 0.05). *Verrucomicrobia* and *Proteobacteria* were enriched with low supplementation of black wolfberry (LAG > NAG; P < 0.05), with no significant difference between NAG and HAG (P > 0.05).

At the genus level (Fig. 8B), *Clostridium*_XIVa, *Megasphaera*, *Pediococcus* and *Wieissella* were more abundant in LAG and HAG samples than in NAG samples (P < 0.05). *Clostridium*_XIVb, *Clostridium*_XVIII, *Butyricicoccus*, *Bifidobacterium*, *Anaerostipes*, *Collinsella*, and *Clostridium* were all enriched in the HAG group compared with the NAG and LAG (P < 0.05). The abundance of *Parabacteroides*, *Akkermansia*, and *Lactococcus* were all higher in the LAG group than in the NAG and HAG groups (P < 0.05). In addition, *Butyricimonas* and *Lactobacillus* were less abundant in NAG than HAG or LAG (P < 0.05); *Fusobacterium*, *Faecalibacterium*, and *Bacillus* were all more abundant in NAG than other groups (P < 0.05); and *Allisonella* was less enriched in HAG compared with LAG and NAG. Interestingly, lactic acid bacteria (LAB), including *Lactococcus*, *Bifidobacterium*, *Lactobacillus*, *Pediococcus*, and *Weissella* were all significantly enriched with addition of black wolfberry extracts (LAG and HAG).

To verify whether SCFAs biosynthesis was indeed enriched following treatment with black wolfberry, we used gas chromatography-mass spectrometry (GC-MS) to quantify SCFAs at 24 h fermentation in NAG, LAG and HAG (Fig. 9). In general, samples with FD black wolfberry had significantly higher contents of acetic acid, propionic acid and butyric acid than NAG at 24 h (LAG and HAG *vs*. NAG; P < 0.05). In addition, HAG had up-regulated trend of acetic acid, propionic acid and butyric acid compared to LAG. Collectively, these findings substantiated the inference that FD black wolfberry has the potential to augment the production of SCFAs within the human gut microbiota.

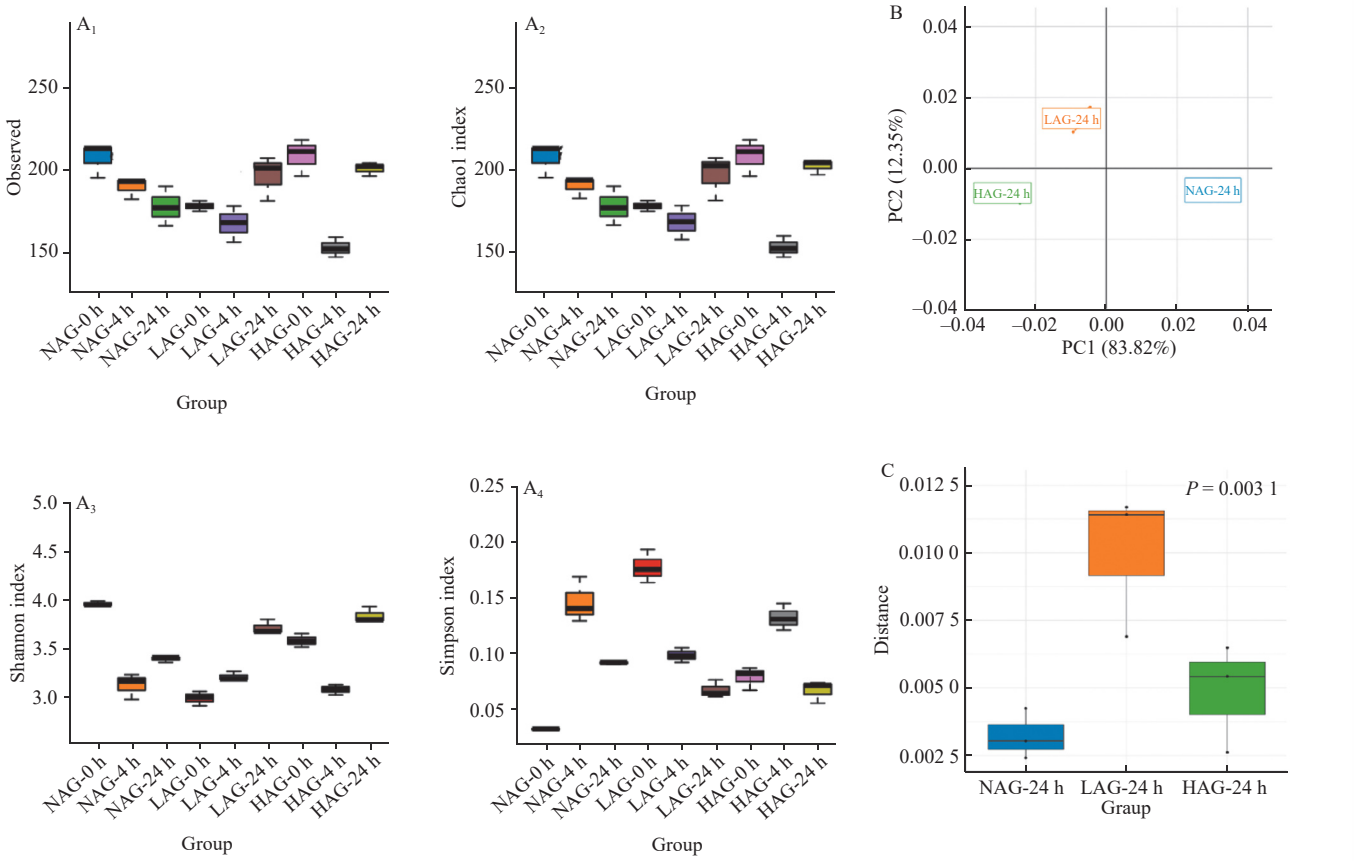

Fig. 6 Analysis of *α*- and *β*-diversity for bacterial communities. (A) *α-D*iversity index; (B) PCoA based on the weighted UniFrac distances of 24 h; (C) PERMANOVA using distance matrices of 24 h.

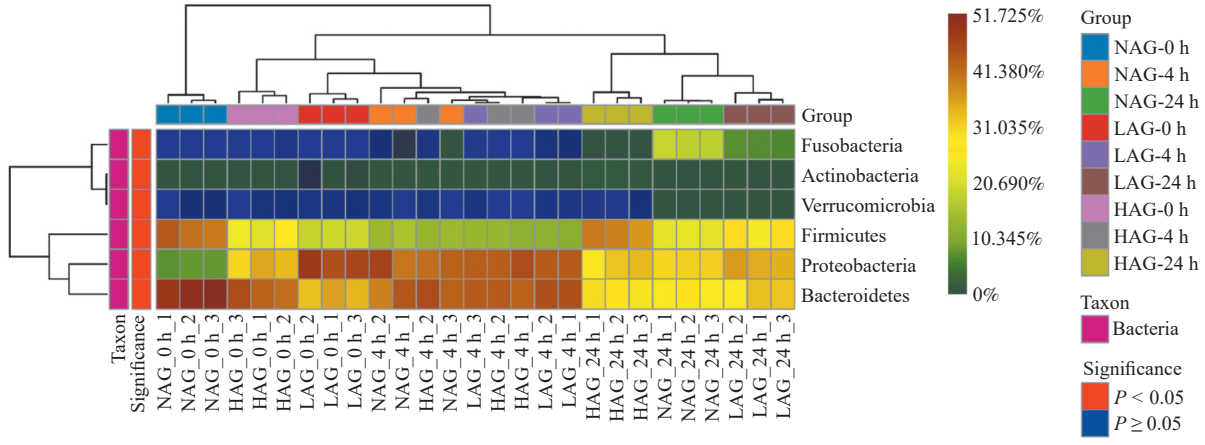

Fig. 7 Heatmap of phylum level analysis of bacterial composition in the different groups during fermentation.

3.5 Up-regulation of fatty acid biosynthesis and downregulation of Lipopolysaccharide biosynthesis in treatments with FD black wolfberry extracts

To investigate whether and which metabolic pathways are enriched under black wolfberry treatment, we used PICRUSt2 with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database to infer functional enrichment in each treatment group. This analysis predicted 160 enriched metabolic pathways based on classification of bacterial OTU sequences. The top 30 most abundant KEGG orthologs that differed among NAG, LAG and HAG groups (Fig. 10) included the significantly down-regulated 'lipopolysaccharide biosynthesis' and 'selenocompound metabolism' pathways. Conversely, the 'pantothenate and CoA biosynthesis', 'pentose phosphate pathway', and 'fatty acid biosynthesis' pathways were markedly up-regulated in the HAG and LAG groups.

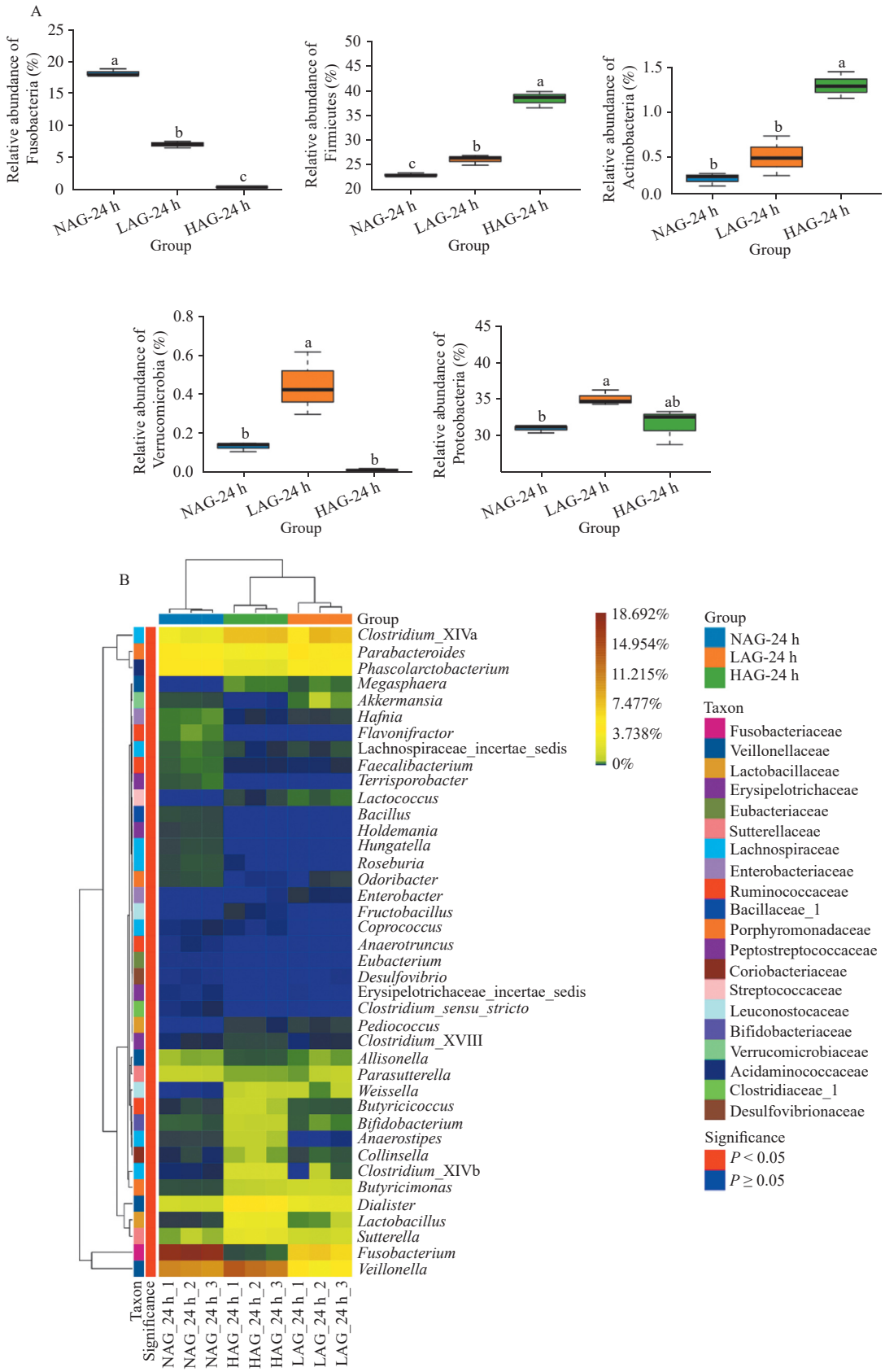

Fig. 8 Analysis of bacterial communities among groups after 24 h fermentation. (A) Boxplot of differential species at phylum level, significant correlations are indicated by *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; **** $P < 0.000\ 1$. (B) Heatmap of NAG, LAG and HAG at genus level.

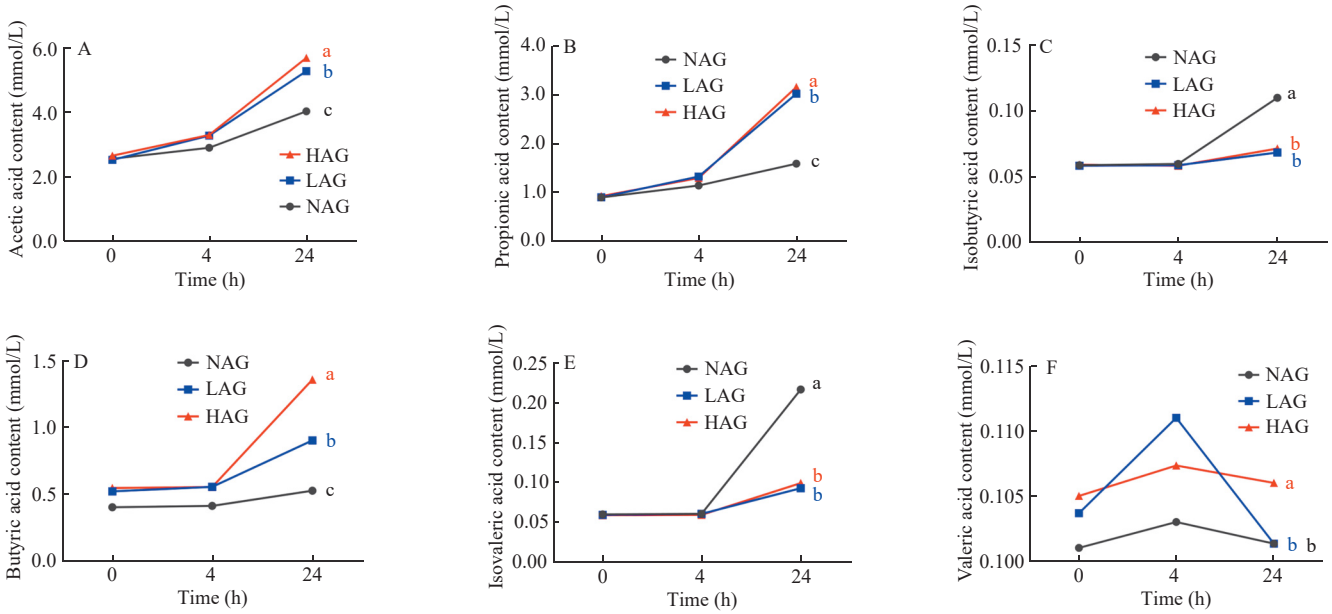

Fig. 9 Profiles of SCFAs by *in vitro* fermentation. (A) Acetic acid; (B) Propionic acid; (C) Isobutyric acid; (D) Butyric acid; (E) Isovaleric acid; (F) Valeric acid. Different lowercase letters indicate significantly different at $P < 0.05$ at 24 h.

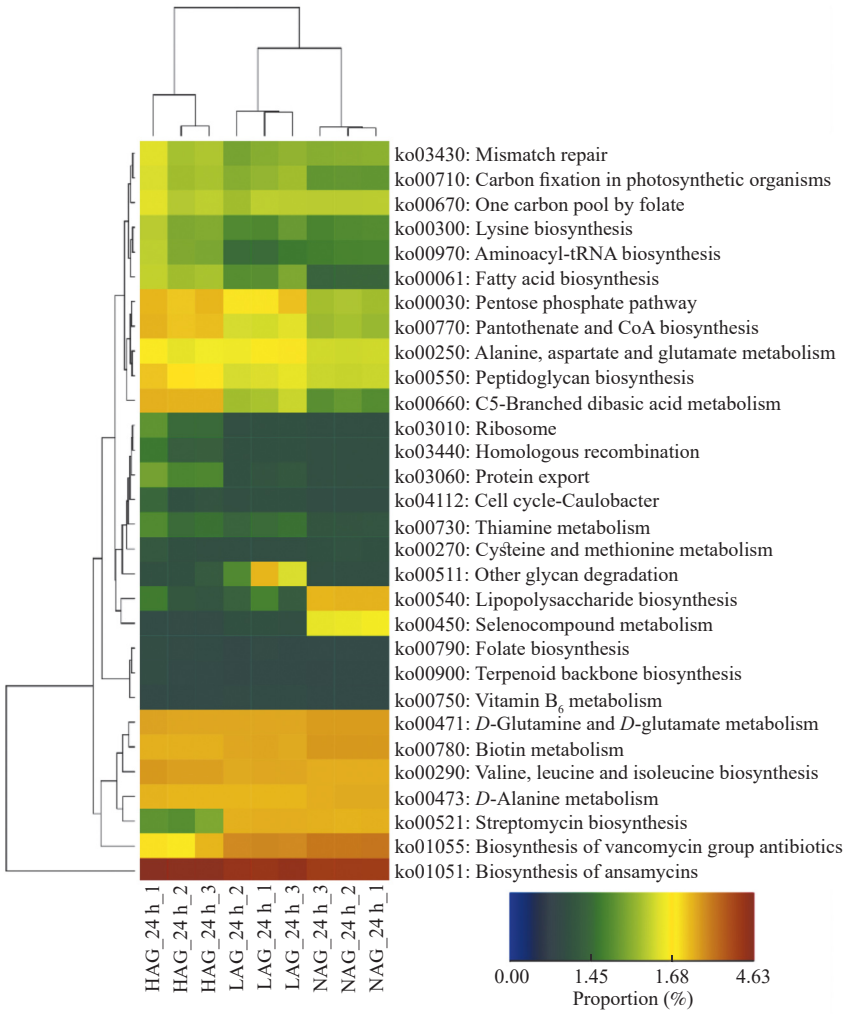

Fig. 10 Heatmap of KEGG orthologs varied among NAG, LAG and HAG groups after 24 h fermentation.

4. Discussion

Black wolfberry is a fruit rich in polyphenols and has been shown to provide various health benefits[35]. Our previous research has revealed that the content of different phenolic compounds in black wolfberry varies depending on its place of origin[26]. However, the influence of black wolfberry drying methods on its phytochemical contents, biological activities, and the effects of dried wolfberry extract on gut microbial community dynamics and composition have yet to be studied. Here, we screened drying methods and compared phenolic metabolites associated with antioxidant and anti-inflammatory effects in black wolfberry produced by each technique. In addition, identified as the optimal drying method, FD black wolfberry was used to further assess the effects on gut microbiota and SCFAs production *in vitro*.

Previous research has shown that black wolfberry berry is rich in polyphenols, condensed tannin content and monomeric anthocyanin content[6]. Our study further revealed that FD black wolfberry samples resulted in higher total polyphenols content, tannins, and proanthocyanidins compared to the samples produced by other drying methods, possibly attributable to the low temperature used for FD that retains the polyphenol metabolites[36]. In addition, vacuum conditions in FD have been suggested to prevent oxidation of polyphenolic compounds by endogenous polyphenol oxidases, which consequently decrease phenolic content[37]. Shih et al.[38] reported that intracellular ice formation during FD resulted in less damage to sweet potato cells (*Ipomoea batatas*) than other drying methods. Proanthocyanidins are a class of polyphenols formed by condensation of flavane-3-alcohol as the main structural unit with typically potent antioxidant properties[39]. In this study, it was observed that the proanthocyanidins content in FD samples significantly surpassed those in all other drying treatments ($P < 0.05$). This notable difference underscored the efficacy of FD in preserving and concentrating proanthocyanidins, suggesting its superiority over alternative drying methods in retaining this particular class of phytochemicals in black wolfberry.

In plants, polyphenols are present in both free and bound states[40], which can modulate properties associated with absorption and utilization in the gastrointestinal tract, thereby affecting their biological function[41]. Profiling the phenolic cations and anions by HPLC-MS quantification in black wolfberry fruits dried under different conditions (Figs. 2 and 3) identified 28 total phenolic, including quercetin-3-*O*-rutinose, naringin, rutin, and narcissoside, which have also been confirmed in previous studies to be common phenolic substances in black wolfberry[4,26]. Comparison of phenolic components *vs*. total polyphenols showed that FD samples had higher contents of bound phenolics than samples from other treatments, while VD samples had the highest contents of free phenolics ($P < 0.05$). This discrepancy could be attributed to the impact of high temperatures during drying processes, potentially leading to the breakdown of phenolic polymers into smaller monomers. As a result, these smaller monomers may be more readily detectable, contributing to the observed increase in free phenolic content and suggesting higher biological accessibility. Dewanto et al.[42] proposed that phenols may undergo oxidation or thermal decomposition during the heating process in drying, and their content could change through enzymatic reactions. The variation in content may also be attributed to the alternating cold and hot processing, potentially promoting the conversion of phenolic substances from the bound state to the free state, resulting in fluctuations in overall phenolic content. Oven rying might result in better availability and separation of phenolic constituents during processing. In a similar vein, Ghafoor et al.[43] observed higher total polyphenols in FD samples, but chromatographic analysis of individual phenolics revealed significantly higher contents in oven-dried ginger[43]. Correspondingly, Kim et al.[44] explored the impact of heat treatment on phenolics in rice hulls and noted that heat treatment led to higher free phenolic acid contents. These collective findings emphasized the multifaceted nature of drying processes and their effects on phenolic composition, considering factors such as oxidation, thermal decomposition, and enzymatic reactions.

Lang et al.[45] found that higher drying temperature can lead to lower total polyphenols contents in black rice and thus influence antioxidant capacity. On the other hand, green tea contains polyphenols that exhibit antioxidant and anti-inflammatory properties, which can potentially offer health benefits by reducing inflammatory signalling pathways[46]. The research revealed that FD black wolfberry samples exhibited heightened antioxidant and anti-inflammatory properties in comparison to other treatments. Notably, a positive correlation was established between FRAP values and the total polyphenols, tannin, and proanthocyanidins contents. This finding underscored the significant influence of phenolics on the antioxidant activity in black wolfberry, aligning with observations in other plant sources such as grape and mulberry leaves[47-48]. Moreover, the results were also similar to Islam et al.[6], dictating that phenolic compounds could be important contributors toward the antioxidant and anti-inflammatory capacities of black wolfberry, phenolic compounds, such as flavonoids, phenolic acids, and condensed tannins are typically recognized as the primary contributors to antioxidant capacity of plants. Additionally, a lower IC_{50} value in the anti-inflammatory trial in this study indicated stronger anti-inflammatory activity, therefore, a negative correlation between IC_{50} values and total polyphenols as well as tannins. FD was determined to be the superior preparation method, demonstrating higher phenolic content and antioxidant activity. However, colorimetric protease inhibitory method, as detailed in Table S1, revealed that the inhibitory rate of all extracts at various concentrations was consistently below 4%. This suggests that the anti-inflammatory effect of *L. ruthenicum* is not attributed to the inhibition of protease activity.

These results underscored the efficacy of FD method in preserving and enhancing the bioactive properties of black wolfberry, further supporting its potential as a valuable source of antioxidants and anti-inflammatory agents, which contributed to the growing body of knowledge regarding optimal processing techniques for maximizing the functional attributes of black wolfberry, with implications for both nutritional science and the development of health-promoting food products.

Bound polyphenols and their derivatives have been identified as prebiotics that enter the gastrointestinal tract and participate in modulating gut microbiota[21]. Morais et al.[49] proposed that polyphenols may be able to selectively enrich probiotic bacterial families like Bifidobacteriaceae and Lactobacillaceae, ultimately changing the composition of gut microbiota. We conducted metagenomic analysis of human gut microbiota fermentations exposed or not exposed to FD black wolfberry *in vitro* to explore the microbial enhancement of FD black wolfberry. The study used an *in vitro* bioreactor that replicates the human gastrointestinal tract, and the results showed that fermentations supplemented with varying amounts of black wolfberry

had higher species richness and diversity, with a distinct microbiota structure compared to untreated samples. Higher microbial diversity can indicate gut resilience characteristic of a healthy state, whereas many human diseases are associated with dysbiosis and decreased microbial diversity in gut microbiota[50].

Characterization of bacterial composition indicated that the Bacteroidetes, Firmicutes, and Proteobacteria are the most prevalent phyla in samples treated with FD black wolfberry, which is consistent with findings of Liu et al.[51] and Zhang et al.[52]. Interestingly, fermentations exposed to black wolfberry showed a decrease in the abundance of Fusobacteria, which has been linked to inflammatory bowel disease[53]. Conversely, the amount of Firmicutes and Actinobacteria increased, suggesting that this supplement may promote the growth of beneficial bacteria. These 2 phyla are major producers of SCFAs, which are crucial for maintaining gut health[54-55]. In addition, fermentations with low levels of black wolfberry were found to enrich Verrucomicrobia and Proteobacteria, which could have a positive impact on human health. Studies have shown that low levels of gut Verrucomicrobia are associated with early hepatocellular carcinoma[56] and progeroid syndromes in mice[57].

Genus level enrichment analysis showed that LAB taxa, such as *Lactococcus*, *Bifidobacterium*, *Lactobacillus*, *Pediococcus* and *Weissella,* were notably enriched in fermentations treated with black wolfberry extracts. LAB are widely accepted as some of the important probiotic organisms for humans, and are purported to promote a diverse range of beneficial effects, such as protection against infectious strains, immunomodulatory effects, mycotoxin degradation, antimicrobial activity, cholesterol reduction, and overall regulation of gut health[58-60]. The most enriched communities in the presence of higher amounts of black wolfberry were *Butyricicoccus* and *Collinsella*. In humans, the production of ursodeoxycholate by *Collinsella* was predicted to have ameliorative effects on symptoms of COVID-19[61]. Research by Cai et al.[62] revealed that polyphenols have the capacity to stimulate the growth of beneficial bacteria such as *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*, leading to the production of SCFAs and a reduction in pH. The butyrate-producing genus, *Butyricicoccus,* can reportedly confer beneficial effects by inhibiting intestinal inflammation and regulating immune function[63-64]. Similarly, another butyrate-producing bacterial genus, *Butyricimonas,* which was enriched in black wolfberry treated fermentations, may contribute to preventing high fat diet-induced diabetes by maintaining function of the intestinal barrier[65]. *Parasutterella,* previously defined as a core fecal microbiome genus in the human gastrointestinal tract[66], was more abundant in high black wolfberry treatments than in untreated controls. By contrast, *Parabacteroides* and *Akkermansia* are enriched in low amounts of black wolfberry (LAG) compared to those in high (HAG) or no (NAG) black wolfberry treatments. Previous work identified a negative correlation between *Parabacteroides* and body mass index[67], while *Akkermansia* appears to competitively inhibit pathogenic bacteria that degrade mucin in the human gastrointestinal tract of humans[68]. Further studies will explore how different dosages of black wolfberry may selectively enrich these potentially beneficial taxa.

Based on functional predictions from PICRUSt2, the inclusion of FD black wolfberry in the diet appears to boost the ‘pentose phosphate pathway’ and ‘fatty acid biosynthesis’ functions within the human gut microbiota. Increased NADPH for nucleotide production and fatty acid biosynthesis that results from these pathways[69], were in accordance with higher SCFAs contents in these fermentations detected by GC-MS, samples treated with FD black wolfberry showed notably higher levels of acetic acid, propionic acid, and butyric acid compared to the NAG group. This finding further suggested that black wolfberry can promote health in the human gut by enhancing microbial and metabolic function. SCFAs can serve as an energy source and substrate for lipid biosynthesis and gluconeogenesis necessary for maintaining the integrity and function of the epithelial barrier[70]. In addition, inferred ‘lipopolysaccharide biosynthesis’ function was significantly lower in fermentations treated with black wolfberry, compared to NAG. Bacterial lipopolysaccharides can provoke an inflammatory immune response, compromise host cell membranes, and encourage antibiotic resistance[71]. The reduction in lipopolysaccharide production, coupled with the boosted anti-inflammatory capacity indicated by the inhibition of NO production in microbiota exposed to black wolfberry, suggests that black wolfberry helps to regulate human gut microbiota to ease inflammatory responses. The observed shifts in microbial richness and diversity, coupled with unique OTUs across different time points and treatments, provide a comprehensive picture of how FD black wolfberry may positively influence the metabolic activities of gut bacteria, ultimately contributing to increased SCFA production. This insight underscores the potential health-promoting benefits of incorporating FD black wolfberry into the diet, highlighting its role in fostering a microbiota-mediated enhancement of SCFA production in the human gut.

Building upon the pivotal conclusion of this study, which highlights the ability of black wolfberry to promote LAB for the reduction of lipopolysaccharide, the research will explore the synergistic potential by combining black wolfberry with the LAB it enhances. This aims to create a biostimulant, investigating the potential synergistic effect. This strategic exploration of combining black wolfberry and LAB holds promise for developing innovative health-promoting interventions that capitalize on their complementary benefits.

5. Conclusion

This study has shown that FD black wolfberry boasts higher phytochemical levels, more robust antioxidant and anti-inflammatory capabilities than other drying methods. Moreover, FD black wolfberry has demonstrated its ability to regulate gut microbiota and microbial metabolic function by promoting the growth of beneficial bacteria and producing SCFAs. These findings represent a significant expansion of our comprehension of the bioactivity and bioactive phytochemicals of black wolfberry, particularly in their intricate interplay with gut bacteria. Moreover, the study provides valuable insights into processing methodologies capable of preserving constituent compounds and antioxidant attributes. While these revelations shed light on the potential benefits of FD black wolfberry, further investigations are imperative to explore its impact on disease models associated with oxidative stress through the intricate interplay between gut microbiota and SCFAs and necessitate additional verification of its efficacy in mitigating lipopolysaccharide-induced effects.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Key Research and Development Program in Xizang Autonomous Region (XZ202301ZY0018N); Xizang Autonomous Region financial special project (XZCZ-SS-2024); the Science and Technology Program of Gansu Province (22JR5RA463); and the Science and Technology Program of Guangdong Province (2023A0505050104).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250233.

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