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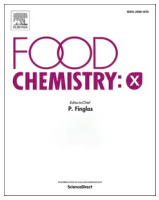

Metabolite profiling, antioxidant and anti-glycemic activities of Tartary buckwheat processed by solid-state fermentation(*SSF*)with *Ganoderma lucidum*

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ABSTRACT

The aim of this study was to investigate the effect of *Ganoderma lucidum* fermentation on antioxidant and anti-glycemic activities of Tartary buckwheat. Xylanase, total cellulase (CMCase and FPase) and β-glucosidase in fermented Tartary buckwheat (FB) increased significantly to 242.06 U/g, 17.99 U/g and 8.67 U/g, respectively. And the polysaccharides, total phenols, flavonoids and triterpenoids, which is increased by 122.19%, 113.70%, 203.74%, and 123.27%, respectively. Metabolite differences between non-fermented Tartary buckwheat (NFB) and FB pointed out that 445 metabolites were substantially different, and were involved in related biological metabolic pathways. There was a considerable rise in the concentrations of hesperidin, xanthotoxol and quercetin 3-O-malonylglucoside by 240.21, 136.94 and 100.77 times (in Fold Change), respectively. The results showed that fermentation significantly increased the antioxidant and anti-glycemic activities of buckwheat. This study demonstrates that the fermentation of *Ganoderma lucidum* provides a new idea to enhance the health-promoting components and bioactivities of Tartary buckwheat.

1. Introduction

Tartary buckwheat is internationally recognized as Liao family of pseudo-cereals with an important part in the human diet, and it is widely distributed in Asia, Europe and the Americas but originated from mountainous provinces of southern China (Huda et al., 2021). Tartary buckwheat is rich in bioflavonoids, chiral inositol, dietary fiber and other functional active ingredients, and possesses anti-glycemic, hypolipidemic, antioxidant and other health effects (Almuhayawi et al., 2021; Xiao, Yang, Xu, Zhang, & Zhang, 2021), thus receiving the attention from a wide range of researchers in the field of food and nutrition. However, Tartary buckwheat cannot be used as a globalized staple food like rice, wheat and other crops because the cell walls of its inner and outer epidermis and endosperm are rich in macromolecules such as pectin, hemicellulose, lignin and cellulose (Zhu, 2020). Therefore, most of the Tartary buckwheat products have a rough texture with particles, excessive consumption can easily cause indigestion, causing intestinal blockage and other problems. In order to improve the quality of Tartary buckwheat, there are researches (Zhang et al., 2021) on enhancing the functional properties of Tartary buckwheat by puffing, crushing and high-pressure techniques. These methods destroyed some of the special active substances inside, reducing its nutritional composition (Li et al., 2022) and due to that it is very difficult to achieve industrialization. Therefore, an urgent theoretical and practical problem to be solved regarding the technical means of improving buckwheat quality still exists.

Fermentation is a technical means of using certain specific functions of microorganisms to biologically modify useful products and thereby increasing their bioactive utilization (Zhang et al., 2022). In recent years, this technology has played an increasingly essential role in the food industry. Tartary buckwheat has special bioactive substances and nutritional properties, which mostly covalently bound to biomacromolecules such as lignin, cellulose, and protein (Zhu, 2020a). They can provide rich nutrients for fungal growth and reproduction and are suitable for Solid-State Fermentation (SSF). *Ganoderma lucidum* is a typical wood-rotting fungus (Wen, Sheng, Wang, Jiang, & Yang, 2022),

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and its mycelium can convert nutrients through its own metabolic activities, which is a source of natural active compounds. Apart from that, it has been used medicinally in China for over 2000 years and is featured with anti-tumour, anti-ageing and anti-oxidant effects (Hayati et al., 2020). It is well known that as the fruiting body consists of highly twisted mycelium, the chemical composition, nutritional value and efficacy of the mycelium are highly similar to those of the fruiting body (Yang et al., 2023). Therefore, the fermentation of Tartary buckwheat with *Ganoderma lucidum* not only preserves the efficacy of *Ganoderma lucidum*, but also improves the nutritional composition of buckwheat. At the same time, the large molecules of Tartary buckwheat can be broken down into smaller ones that are more easily absorbed by the body, thus reducing its coarse taste. It has been shown that fungal fermentation technology significantly improves the active substances of processed products. By using fungal solid-state fermentation of mulberry leaves and buckwheat, respectively, it was found that during fermentation, microorganisms secrete a large number of hydrolytic enzymes which increasing the total phenolic and flavonoid content, thereby improving the antioxidant capacity of buckwheat and α-glucosidase inhibitory activity (Xiao et al., 2021; Xiao, Yang, et al., 2021; Zhao, Yan, Yue, Yue, & Yuan, 2023). In addition, fungal fermentation can substantially reduce the antinutritional factors such as mustard, thioglucoside and phytic acid in agricultural by-products such as rapeseed meal, soybean residues and wheat bran (Tuly et al., 2022). It can also degrade fiber and lignin (Heidari et al., 2022), converting agricultural waste into high value-added products, because the hydrolytic enzymes (i.e. β-glucosidase, xylanase, cellulase and α-amylase) secreted by fungi during fermentation can hydrolyze ethers, esters or glycosidic bonds (Xiao et al., 2021), breaking down the fermentation substrate into smaller molecules (e.g. polysaccharides into reducing sugars, proteins into small peptides, amino acids, etc.), thus enhancing the biological activity of the fermentation substrate and achieving high value utilization.

Therefore, in this study, Tartary buckwheat was inoculated with *Ganoderma lucidum* for fermentation. The effect of *Ganoderma lucidum* fermentation on the bioactive substance content of Tartary buckwheat was determined. Then, the mechanism was clarified through the structural characteristics of the fermentation products and the activity of carbohydrate-hydrolyzing enzymes. In addition, Ultra-performance liquid chromatography (LC)–mass spectrometry (MS)-based untargeted metabolomics was performed to elucidate the complex metabolite changes that occurred during the production on the antioxidant capacity and anti-glycemic effects of Tartary buckwheat. Besides, it enriches the theoretical basis and provides a scientific reference for the industrial production of fermented Tartary buckwheat with *Ganoderma lucidum*.

2. Materials and methods

2.1. Materials

Trolox, DPPH, ABTS, p-nitrophenol, *p*-nitrophenyl-β-D-glucoside (PNPG), DNS, α-amylase, α-glucosidase, xylanase and cellulase were obtained from Macklin Biochemical., Ltd. and Solarbio Biochemical., Ltd. (Shanghai, China). All other chemicals and reagents, such as anhydrous sodium carbonate, potassium ferricyanide, salicylic acid, potassium persulphate, methanol, acetone and anhydrous ethanol used in this study were of analytical grade. Tartary Buckwheat was purchased from Dongfang Shengu Co., Ltd. (Guizhou, China). The fungus *Ganoderma lucidum* (G10) acquired from Minyuan Fungus (Chongqin, China) was used as a starter for the processing of fermented Tartary buckwheat, stored on PDA agar slant and subcultured every month. Beyond that, *Ganoderma lucidum* (G10) slants were incubated at 26 °C for 7 days and subsequently preserved in a refrigerator at 4 °C.

2.2. SSF of Tarty buckwheat

500 g of Tartary buckwheat should be prepared by soaking it in 1% calcium hydroxide solution for three hours, followed by draining excess water, adding 30% (*w*/w, based on total Tartary buckwheat sample) wheat bran and food-grade lime in order to adjust the pH to 9–10 with pH paper, and then mixing well for a solid medium. Pack the medium into 12 × 24 × 5 cm polypropylene bags, with 200 g of medium per bag. After that, autoclaved at 121 °C for 90 min. After the sterilized Tartary buckwheat had cooled to room temperature, two to three pieces of activated *Ganoderma lucidum* (G10) strains (1 × 1 cm^2) were inoculated in the middle of the medium. It was then placed at 26 °C and allowed to ferment for 21 days in a microbiological incubator. A blank control was also made, and buckwheat was not inoculated with *Ganoderma lucidum* under the same conditions. The FB and NFB were freeze-dried separately, crushed in a high-speed rotary grinder and then passed through an 80-mesh sieve to obtain the fine powder. After that, samples were stored in a refrigerator at 4 °C until further analysis. The FB and NFB were made into triplicates.

2.3. Carbohydrate-hydrolyzing enzyme activity assay

The enzyme solution was prepared following the method reported by Xie et al. (2021). FB and NFB were added to citric acid buffer solution (0.1 M, pH 4.8) at a liquid to material ratio of 1:50 (M/W). After that, shake in a water bath shaker at 28 °C and 120 rpm for 2 h. After centrifugation at 8000*g* (4 °C) for 10 min in a GTR318A refrigerated centrifuge (Hunan Kecheng Instrument Equipment Co., Ltd., China), the supernatant was collected as the crude enzyme solution. Moreover, the activities of β-glucosidase, total cellulase (such as FPase: paper cellulase activity and CMCase: carboxymethyl cellulase activity), and xylanase were then determined. The total cellulase activities and the activity of filter paper enzyme were determined by mixing 1 mL of crude enzyme solution with 50 mg of filter paper (Whatman No. 1). Then cut into 1 × 6 cm strips, and add 1 mL of 2% carboxymethyl cellulose and 1 mL of citric acid buffer solution, respectively, followed by incubating at 40 °C for 30 min. At the end of the reaction, the mixture was heated in boiling water for 5 min in order to inactivate the enzymes, and then the glucose concentration in 1 mL of supernatant was determined using a Microplate Reader (ZDM-1101 ELISA, China) at the wavelength of 540 nm. The xylanase activity was similar to the above reaction, with 2% xylan as substrate and an incubation temperature of 50 °C. In contrast, the activity of β-glucosidase was assessed by incubating 2 mL of crude enzyme solution with 5 mM *p*-nitrophenyl-β-D-glucopyranoside (PNPG) 2 mL at 50 °C for 30 min, followed by inactivation of the enzyme for 5 min in boiling water. To stop the reaction, the supernatant was combined with 15% sodium carbonate, and the amount of p-Nitrophenol released was determined by a Microplate Reader at 400 nm. One unit of β-glucosidase, cellulase, filter paper enzyme, and α-amylase activity corresponds to the amount of enzyme that hydrolyses the respective substrate to release 1 μmol equivalent of product per minute and is expressed as U/g.

2.4. Scanning Electron Microscope(SEM)

To compare scanning electron microscopy (SEM) images of FB and NFB structures, an S-3400 N scanning electron microscope (HITACHI (Hitachi), Japan) was adopted to obtain external and internal images at 100, 1000 and 2000× magnification, respectively, at an accelerating voltage of 5 kV. The samples were freeze-dried in advance.

2.5. Determination of active substances

2.5.1. Extraction preparation

1 g of NFB and FB powder was thoroughly mixed with methanol (*w*/*v*, 1:50) in an Erlenmeyer flask. The extracts were sonicated at 40 °C for 30 min followed by being centrifuged at 10,000 ×*g* (4 °C) for 15 min to obtain the supernatant. The residue was re-extracted twice under the same conditions. After centrifugation, all collected supernatants were concentrated at 40 °C using a vacuum evaporator (Biochemical

Instrument Factory of Shanghai Yarong, China). After that, the methanol was fixed to 25 mL and stored in a refrigerator at −4 °C until use.

2.5.2. Determination of total triterpenoids

According to Hsu, Chen, Lai, & Chen (2021) with slight modifications, 0.3 mL of methanol extract was pipetted in a 10 mL test tube; the tube was put into a 90 °C water bath to evaporate the solvent. Then, 0.1 mL 5% vanillin-glacial acetic acid and 0.8 mL perchloric acid were added, mixed thoroughly and redissolved at 60 °C. After a 20 min incubation, it was cooled in an ice bath for 3–5 min, followed by 5 mL glacial acetic acid being added, mixed well and left at room temperature for 10 min. The absorbance was measured at 550 nm under the UV-2600 spectrophotometer (Beijing, China). The total triterpenoid content was calculated from the absorbance value to the regression line of the oleanolic acid standard curve.

2.5.3. Determination of total phenolic content

The method was slightly modified by referring to Zhang, Ye, & Tan (2022). To be specific, 250 μL of the extract and 500 μL of distilled water were accurately pipetted and mixed; afterwards, 1 mL of 0.2 mol/L of forinol reagent was added and shaken well. Then, the incubation was carried out at 30 °C for 30 min. After that, 2 mL of 10% sodium carbonate solution was added and the mixture was incubated at 30 °C for 30 min, followed by the absorbance being measured at 760 nm. The total phenolic content was calculated from a standard gallic acid curve, and the results are presented as milligrams of gallic acid equivalent (GAE) per 100 g of Tartary buckwheat dry weight (mg GAE/100 g DW).

2.5.4. Determination of total flavonoid content

The total flavonoid content was determined by the method with slight modifications (Chaitra, Abhishek, Sudha, Vanitha, & Crassina, 2020). Briefly, 1 mL of the extract was taken up in a 10 mL volumetric flask, and then, $AlCl_3$ solution (2 mL, 0.1 mol/L) and potassium acetate solution (3 mL, 1 mol/L) were added separately; finally, the mixture was fixed with methanol to the mark and shaken well. Here, it should be noted that the mixture was allowed to stand at room temperature for 60 min and the absorbance was measured at 420 nm. The flavonoid content was expressed as milligrams of quercetin equivalent (QE) per 100 g of Tartary buckwheat dry weight (mg QE/ 100 g DW).

2.6. Determination of polysaccharide content

The extraction was conducted according to the method adopted by Hsu et al. (2021) with slight modifications. A 0.5 g sample was weighed, added to 20 mL of anhydrous ethanol and extracted by ultrasonication for 30 min. After extraction, the sample was centrifuged at 4000 r/min for 10 min, and the supernatant was discarded. The insoluble material was washed with 10 mL of ethanol (80%) and centrifuged. The insoluble material was transferred to a round bottom flask with deionized water; 50 mL of distilled water was added, and the extraction was carried out in a boiling water bath for 2 h. The sample was cooled to room temperature, filtered and fixed at 100 mL. After that, 1 mL of the sample solution was taken in a test tube, followed by 1 mL of phenol solution (5%) and 5 mL of concentrated sulphuric acid in turn, shaken well and left to stand for 10 min. Then, the absorbance was measured at 490 nm. The total polysaccharide content was calculated from the absorbance value to the regression line of the glucose standard curve.

2.7. Extraction of metabolite and LC-MS/MS analysis

FB and NFB samples (100 mg) were prepared in a centrifuge tube, then mixed thoroughly with 1 mL of 70% methanol water using a vortex stirrer and centrifuged at 12000 r/min for 10 min at 4 °C. After that, 150 μL of supernatant was pipetted into another centrifuge tube and allowed to stand for 30 min in a refrigerator at −20 °C. The supernatant was then centrifuged at 12000 r/min for 3 min at 4 °C and injected to an LC-MS/MS system (Shimadzu LC-30 A, Japan) for metabolite analysis.

Metabolites in fermented and non-fermented Tartary buckwheat extracts were separated and identified using an ultra-high-performance liquid chromatography (Shimadzu LC-30 A, Japan) composed of the vacuum degasser, binary pump, autosampler and column oven connected to an AB Triple TOF 6600 mass spectrometer. The analytical column used was a Waters Acquity UPLC BEH C18 column (1.8 μm, 2.1 × 100 $mm^{2)}$. The separation conditions were as follows. Ultrapure water (containing 0.1% formic acid) was taken as eluent A, and acetonitrile (containing 0.1% formic acid) was used as eluent B for the positive polar mode. Ultra-pure water (5 mM ammonium formate, pH 9.0) was selected as eluent A, while acetonitrile (containing 0.1% formic acid) as eluent B in the negative polar mode. The total separation time for chromatographic analysis was set at 14 min. The gradient elution program was initiated in 5% B and then increased from 0 min to 11 min. Eluent B was ramped up to 90% with a linear gradient and held for 1 min, followed by being returned to 5% in 0.1 min and held for 1.9 min. A solvent flow rate was 0.4 mL/min during the above gradient elution. Furthermore, Triple TOF 6600 mass spectrometer was carried out in the ESI positive and negative mode.

2.8. Antioxidant activity assay

The effect of antioxidant activity of fermented buckwheat from *Ganoderma lucidum* was studied using OH^- radical scavenging capacity, reducing power (RP), and scavenging activities of $ABTS^{\bullet+}$ and DPPH radicals. The DPPH radical scavenging effect and $ABTS^{\bullet+}$ scavenging capacity of fermented and non-fermented Tartary buckwheat extracts were studied by following the method reported by Zhang et al. (Zhang, Zhou, et al., 2022) after slight modifications. A standard curve was made using Trolox as an equivalent, where y is the absorbance and x is the Trolox molar concentration (mmol/L), and besides, it was also plotted employing different concentrations of Trolox standard solutions. The reducing power and OH^- radical scavenging capacity ability was determined according to the method adopted by Hsu et al. (Hsu et al., 2021), with Trolox as the positive control and the reducing power of the sample expressed as the equivalent of Trolox standard (μmol Trolox/ 100 g DW) in 100 g of Tartary buckwheat dry weight.

2.9. Determination of α-amylase and α-glucosidase inhibitory activity

2.9.1. Determination of α-amylase inhibitory activity

α-amylase inhibitory activity was determined according to the method used by Xie et al. (2021) with slight modifications by drawing 200 μL of the above extract and mixing it with 200 μL of α-amylase (1 U/ mL). Subsequently, 1 mL of 1% soluble starch was added and incubated for 10 min. After that, 0.4 mL of DNS was added and a boiling water bath was used for 5 min, followed by the addition of 2 mL of deionized water and scanning at 540 nm using the Microplate Reader. Here, it should be mentioned that all these reaction solutions were pre-incubated at 37 °C for 10 min.

$$I\,(\%) = 1 - (A3 - A2)/(A1 - A0) \times 100\%$$

A0 is the control blank group; A1 refers to the control reaction group; A2 denotes the sample blank group, and A3 represents the sample reaction group.

2.9.2. Determination of α-glucosidase inhibitory activity

Here is a slight modification from the method of Xie et al. (2021). 100 μL of 1.0 U/ mL of α-glucosidase solution (prepared with pH 6.9 0.1 M phosphate buffer) and 100 μL of extract were mixed well in a centrifuge tube for the reaction. Subsequently, 200 μL of PNPG (5 mM, prepared with pH 6.9 0.1 M phosphate buffer) was added and the reaction was terminated by adding 200 μL of 1 moL sodium carbonate solution in a water bath at 37 °C for 30 min, followed by scanning at 405

nm.

$$I\ (\%) = 1 - (A3 - A2)/(A1 - A0) \times 100\%$$

A0 is the control blank group; A1 refers to the control reaction group; A2 denotes the sample blank group, and A3 represents the sample reaction group.

2.10. Data analysis

All experiments were performed independently and in triplicate. The experimental data obtained were expressed as mean ± standard deviation (SD). One-way ANOVA and Duncan's multi-factor test were conducted using SPSS 26.0 software to determine significant differences between means ($p < 0.05$). Furthermore, the graph of data for different evaluated antioxidant activities, anti-glycemic properties etc. was depicted adopting OriginPro 2022 statistical software (OriginLab Co., USA).

3. Results and discussion

3.1. Determination of carbohydrate-hydrolyzing enzymes after Tartary buckwheat fermentation

In order to explore the effect of SSF on the bioactive substances of Tartary buckwheat, the hydrolytic enzymes cellulase, β-glucosidase and xylanase secreted by the fungi were measured in this study. Cellulose and xylan are macromolecular polysaccharides composed of glucose, which can be degraded by carbohydrate hydrolases such as cellulase, β-glucanase and xylanase (Bei, Chen, Lu, Wu, & Wu, 2018). As shown in Table 1, the key hydrolytic enzymes produced by *Ganoderma lucidum* during solid-state fermentation, β-glucosidase, CMCase, xylanase and FPase were 242.06 U/g, 13.05 U/g, 8.67 U/g and 4.94 U/g, respectively. Moreover, total cellulase activity (CMCase and FPase) was not detected in NFB, but the other two enzyme activities both were 3.31 and 4.49 times higher than that before fermentation.

These results indicated that during the SSF of Tartary buckwheat, with the consumption of the substrate for carbon hydrolase and the accumulation of cellulose during the fermentation period, higher β-glucosidase activity (Xiao, Yang, et al., 2021) appeared, with CMCase and xylanase activity at intermediate levels, and relatively low FPase activity. During the SSF process, *Ganoderma lucidum* promoted the production of cellulases. Xylanase and ß-glucosidase, as important hydrolytic enzymes for microorganisms (especially filamentous fungi), hydrolyze glucan and xylan into glucose and xylose to provide nutrients for the growth of *Ganoderma lucidum* and facilitate the growth of mycelium, thus increasing the bioactive substances (polysaccharides, triterpenes, total phenols and flavonoids, etc.) in the fermentation products. It was reported that extensive filamentous fungi can produce extracellular enzymes such as cellulases, lignases and β-glucosidases during fermentation, which can degrade the linkages between phenolic substances and structural components of the cell wall, leading to the release of soluble phenolic substances (Zhang, Lu, & Liu, 2022). This is similar to the report of (Zhao et al., 2023) that hydrolytic enzymes (cellulase, xylanase, etc.) secreted by SSF of *Pleurotus ostreatus* were used to participate in the breakdown concerning the dermis of mulberry leaf cells, thus improving the composition and content of polyphenols and flavonoids. Thus, the hydrolytic enzymes produced by *Ganoderma lucidum* are closely related to the disintegration of buckwheat cell walls for the purpose of enhancing the bioactivity in fermented buckwheat.

Table 1
Carbohydrate-hydrolyzing enzymes activity of non-fermented Tartary buckwheat and *Ganoderma lucidum*-fermented.

Carbohydrate-hydrolyzing enzymes	NFB	FB
CMCase activity(U/g)	ND	13.05 ± 0.41
FPase activity(U/g)	ND	4.94 ± 0.04
β-Glucosidase activity(U/g)	73.06 ± 4.79[a]	242.06 ± 79.67[b]
Xylanase activity(U/g)	1.93 ± 0.03[a]	8.67 ± 0.17[b]

Values were expressed means ±standard deviation of three determinations. ND, no detected. Values followed by different superscript small letters (a–b) are remarkably different ($p < 0.05$) among the NFB and FB.

3.2. SEM analysis

The images of Tartary buckwheat fermentation, non-fermentation and untreated buckwheat are shown in Fig. 1. Figs. A1, B1 and C1 display micrographs of untreated Tartary buckwheat (UTB), non-fermented Tartary buckwheat (NFB) and fermented Tartary buckwheat (FB) at 100×, whereas Figs. A2, B2 and C2 present micrographs of UTB, NFB and FB at 1000×; and Figs. A3, B3 and C3 show micrographs of UTB, NFB and FB at 2000×. A clear continuous structure was observed in the UTB, with a smooth surface and a tight texture, closely bound to the starch granules. In contrast, the NFB showed slight bulging, and at 1000×, fine particles of fiber were seen to expose on the surface. In this case, it is shown that the lignin on the fiber surface is destroyed to some extent during the sterilization process (Lin et al., 2021), and the tight structure of the buckwheat cell wall fiber network is disrupted, leading to cell lysis and the release of more substances such as phenols and polysaccharides, which are lost due to their instability at high temperatures (Li et al., 2023). The microstructure under FB was obviously different from the previous two, with the FB having a rougher, looser texture and a large number of stomata and furrows, indicating that the *Ganoderma lucidum* mycelium had invaded the internal matrix of buckwheat and consumed a large amount of hemicellulose and lignin during growth, exacerbating the damage to the buckwheat cell wall. During the fermentation process, *Ganoderma lucidum* produced many hydrolytic enzymes, such as cellulase, xylanase and β-glucosidase, which degraded the fiber components of the cell wall, promoted the penetration and release of internal substances (Lu, Li, Zhou, Hu, & Liu, 2022) and improved its biological activity.

3.3. Determination of active substances after Tartary buckwheat fermentation

As the polysaccharides, phenolics, flavonoids and triterpenoids in the plant mechanism after fermentation of Tartary buckwheat by *Ganoderma lucidum* are natural antioxidants, they have received a lot of attention from researchers. The hydrolytic enzymes (i.e., β-glucosidase, xylanase, cellulase) produced by the *Ganoderma lucidum* fermentation are closely related to the content of active substances in Tartary buckwheat. These enzymes can hydrolyze ethers, esters or glycosidic bonds, effectively breaking down the insoluble substances in the cell wall and releasing them from the cell wall, thus strengthening the biological activity of the grain (Xiao, Huang, et al., 2021). In this study, the active substances in NFB and FB were extracted and determined, and the results are shown in Table 2. It was found that the active ingredients of FB were significantly higher than those of NFB. Polysaccharides and triterpenoids increased by 122.19% and 113.70%, respectively, and flavonoids and total phenols also rose by 2.04 and 1.14 times, accordingly. Previous studies have demonstrated that only a small number of phenolic compounds exist in the form of free esters, while most phenolics are covalently bound to cellulose, hemicellulose, pectin and other components of the cell wall in an insoluble bound form (Xie et al., 2021), which can only be hydrolyzed and extracted under alkaline conditions and high temperatures. However, bound phenols increased from 96.47 mg GAE /100 g DW to 231.97 mg GAE /100 g DW after SSF. This was attributed to the cellulase activity and β-xylanase activity secreted by *Ganoderma lucidum* during fermentation increased to a great extent, which can degrade the ether bonds formed by bound phenols with lignin and the ester bonds formed with structural carbohydrates and proteins, thus promoting the bound phenol release, making them easy to extract

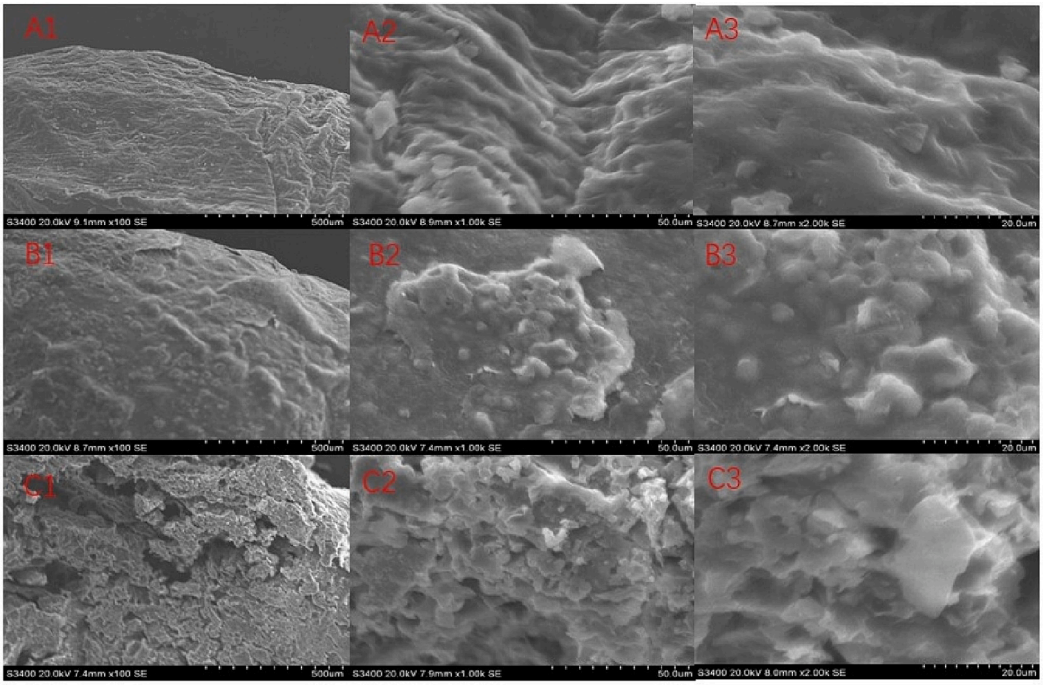


Fig. 1. Effect of *Ganoderma lucidum* fermentation on the surface microstructure of tartary buckwheat. A, The surface microstructure of UTB; B, The surface microstructure of NFB; C, The surface microstructure of FB.

Table 2
Bioactive components of non-fermented Tartary buckwheat and *Ganoderma lucidum*-fermented.

Bioactive components	NFB	FB
Polysaccharide(mg/100 g)	5660.04 ± 396.49[a]	12,576.25 ± 1066.88[b]
Triterpene(mg/100 g)	400.45 ± 21.31[a]	963.77 ± 101.41[b]
Free Phenol (mg GAE/100 g DW)	164.71 ± 7.46[a]	326.2 ± 2.83[b]
Bound Phenol (mg GAE/100 g DW)	96.47 ± 8.98[a]	231.97 ± 12.86[b]
Total Phenol (mg GAE/100 g DW)	261.19 ± 16.1[a]	558.17 ± 15.69[b]
Flavonoids (mg QE/100 g DW)	534.97 ± 39.92[a]	1624.89 ± 48.62[b]

Values were expressed means ±standard deviation of three determinations. ND, no detected. Values followed by different superscript small letters (a–b) are remarkably different ($p < 0.05$) among the NFB and FB.

at room temperature (Bei et al., 2018). This is consistent with the report of Xiao, Yang, et al. (2021) who significantly increased the phenolic content and further improved antioxidant activity by using *Eurotium cristatum* YL-1 to ferment buckwheat.

The increase in polysaccharide content from 5660.04 mg/100 g to 12,576.25 mg/100 g after fermentation was attributed to the secretion of large amounts of cellulase and xylanase by *Ganoderma lucidum*. These hydrolytic enzymes hydrolyze the polymers consisting of xylan, cellulose and lignin together, which are mainly present in the secondary cell wall of buckwheat, into monosaccharides (Bei et al., 2018). Thus, providing a rich carbon source material for *Ganoderma lucidum* mycelium to promote mycelial growth, thereby increasing the crude polysaccharide content in the mycelial fermentation product (Hsu et al., 2021). Ganoderma triterpenes are one of the main medicinal components of *Ganoderma lucidum* and an important index to measure its economic value. Triterpenes are present in buckwheat as phytosterols, ergosterols, ganoderic acid and other bioactive substances (Li, Gu, et al., 2023). After fermentation, the triterpenoid content reached 963.77 mg/100 g, because the cellulase secreted by the mycelium of *Ganoderma lucidum* degraded the buckwheat cell wall, effectively activating and releasing the phytosterols, ergosterol, ganoderic acid and other active substances in the cell wall, thus increasing the triterpenoid content of Tartary buckwheat, which was in line with the activity of carbon hydrolase.

These results suggest that fungal fermentation can increase the bioactive substances of Tartary buckwheat. The results observed in this experiment are consistent with the report of Xiao, Huang, et al. (2021) that the SSF of black beans by *Eurotium cristatum* YL-1 substantially increased the nutritional value (including amino acids, minerals, fatty acids, total phenols, and isoflavones), flavor characteristics and antioxidant activity. So SSF is a technological tool that can give high-added value to agricultural products and their by-products.

3.4. Metabolic analyses of Tartary buckwheat treated by SSF with *Ganoderma lucidum*

3.4.1. PCA analyses

In the unsupervised model, PCA was made to assess the overall differences between the FB and NFB samples. In the PCA plot, each sample in each group was represented by a single point, and the differences between samples were indicated based on the trend of aggregation and separation of samples in the plot, where more aggregated points indicated greater similarity in the observed variables, and the greater dispersion of points denoted more significant differences in the observed variables (Zhan et al., 2023). The PCA plots of the relationships between NFB and FB samples are shown in Fig. 1. The first principal component PC1 (63.5%) and the second principal component PC2 (24.9%) together explained 88.4% of the total variance. The FB and NFB samples were highly concentrated at all points on the sample plot, indicating a high degree of reproducibility in the collection process. More importantly, the buckwheat samples before and after fermentation were distributed in different areas, with a significant distance between the FB and NFB samples, suggesting that fermentation of Tartary buckwheat with *Ganoderma lucidum* can dramatically alter the composition of metabolites (Fig. 2).

3.4.2. Analysis of differential metabolites during Tartary buckwheat fermentation

The PCA scoring plots between the FB and NFB groups showed obvious separation, revealing that the metabolite differences were

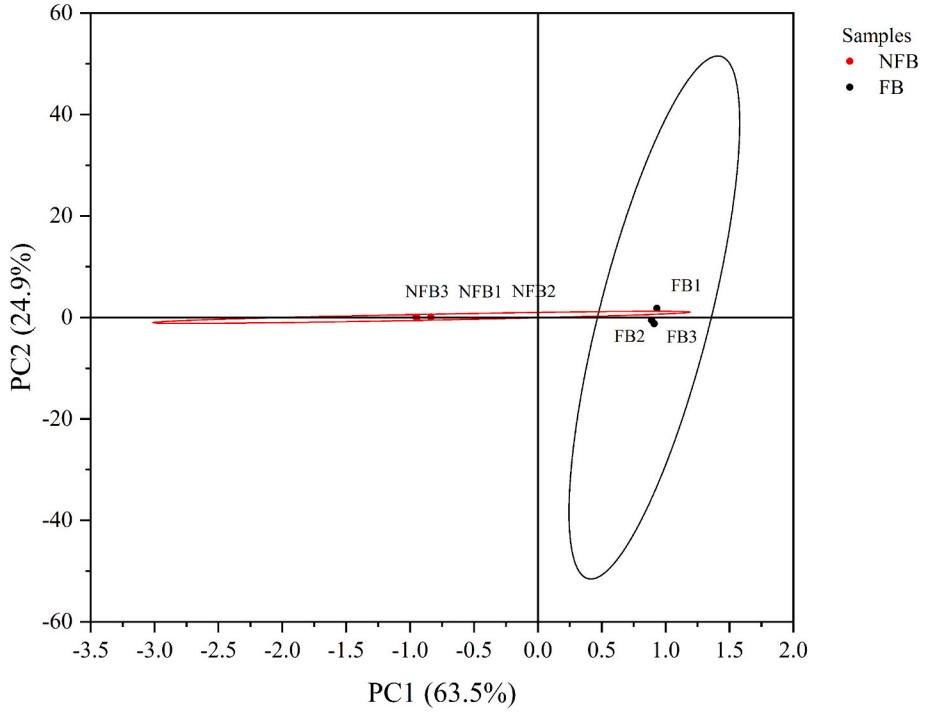


Fig. 2. The total sample Principal component analysis (PCA) of FB and NFB samples.

remarkable. Then, the differential metabolites were initially annotated and identified by comparing with retention times (RT), molecular formulae, accurate mass data, multiple databases of MS/MS spectra, reported literature references and public databases (e.g., Metlin, HMDB and mzCloud) as well as by excluding isomers that could not be accurately distinguished. By merging the results of $p < 0.01$ and Fold Change (FC) < 0.5 or > 2, the differential metabolites between FB and NFB were screened. To be specific, 544 distinct metabolites, including carbohydrates and their derivatives, alkaloids, terpenoids, organic acids and their metabolites, amino acid derivatives, and ketones, were involved. As for the metabolites, they were classified into 22 categories (Fig. 3A), with the most diverse metabolites reflecting the diversity of the SSF process being organic acid and its derivatives (14.15%), amino acid and its metabolites (9.93%), flavonoids and phenols (9.38%) and carbohydrate and its metabolites (6.43%). For the purpose of efficiently describing the changes in metabolites and the various maps of expression level during fermentation, the full samples obtained from the UPLC-MS spectra of NFB and FB in positive and negative modes were examined. Volcano plots can clearly depict the significance concerning differential expression of metabolites in different sample groups. As displayed in Fig. 3B, 228 metabolites were significantly up-regulated, while 99 metabolites were not dramatically changed, and 217 metabolites were obviously down-regulated.

In this study, in order to further identify the differences between the main metabolites, organic acids, amino acids, flavonoids, phenols and carbohydrates were analyzed by cluster thermography. Organic acids are the intermediate or final products of cellular metabolism. As shown in Fig. 4A, compared with NFB, 39 kinds of organic acids including glutaric acid, gluconic acid 6-phosphate, citric acid, oxoglutaric acid and succinic acid were significantly upregulated ($p < 0.01$, $Log_2FC > 2$).

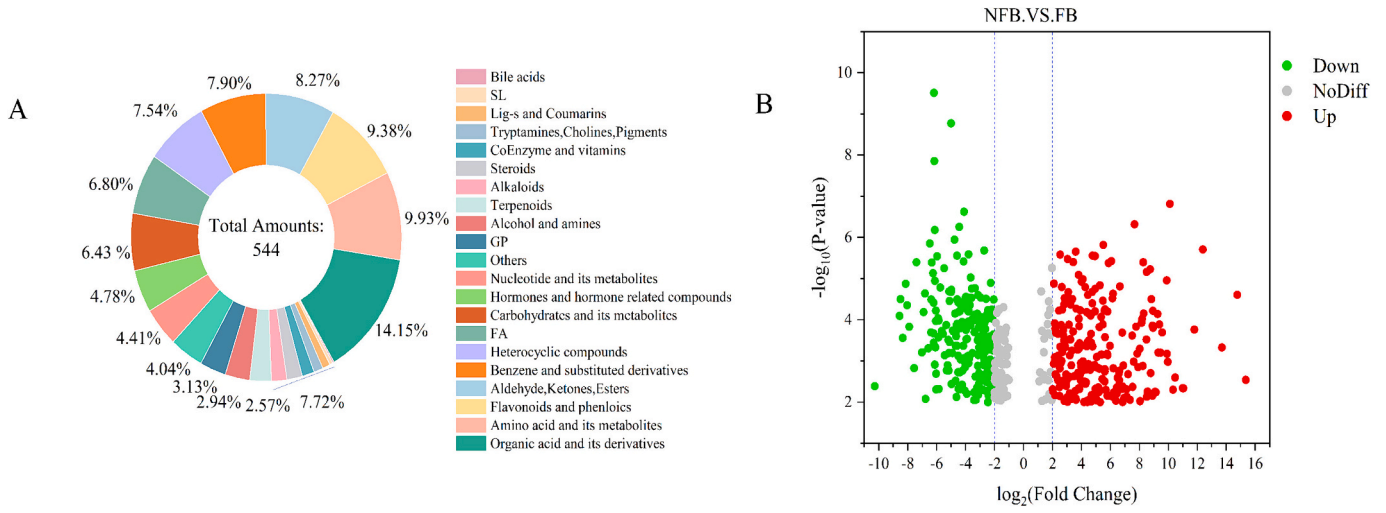


Fig. 3. A, Classification of all the detected metabolites of NFB and FB samples. B, Volcano plot of the differential metabolites of NFB vs. FB. Each dot represents one metabolite. UP, up-regulated compounds; Down, down-regulated compounds; No Diff, no significantly different compounds.

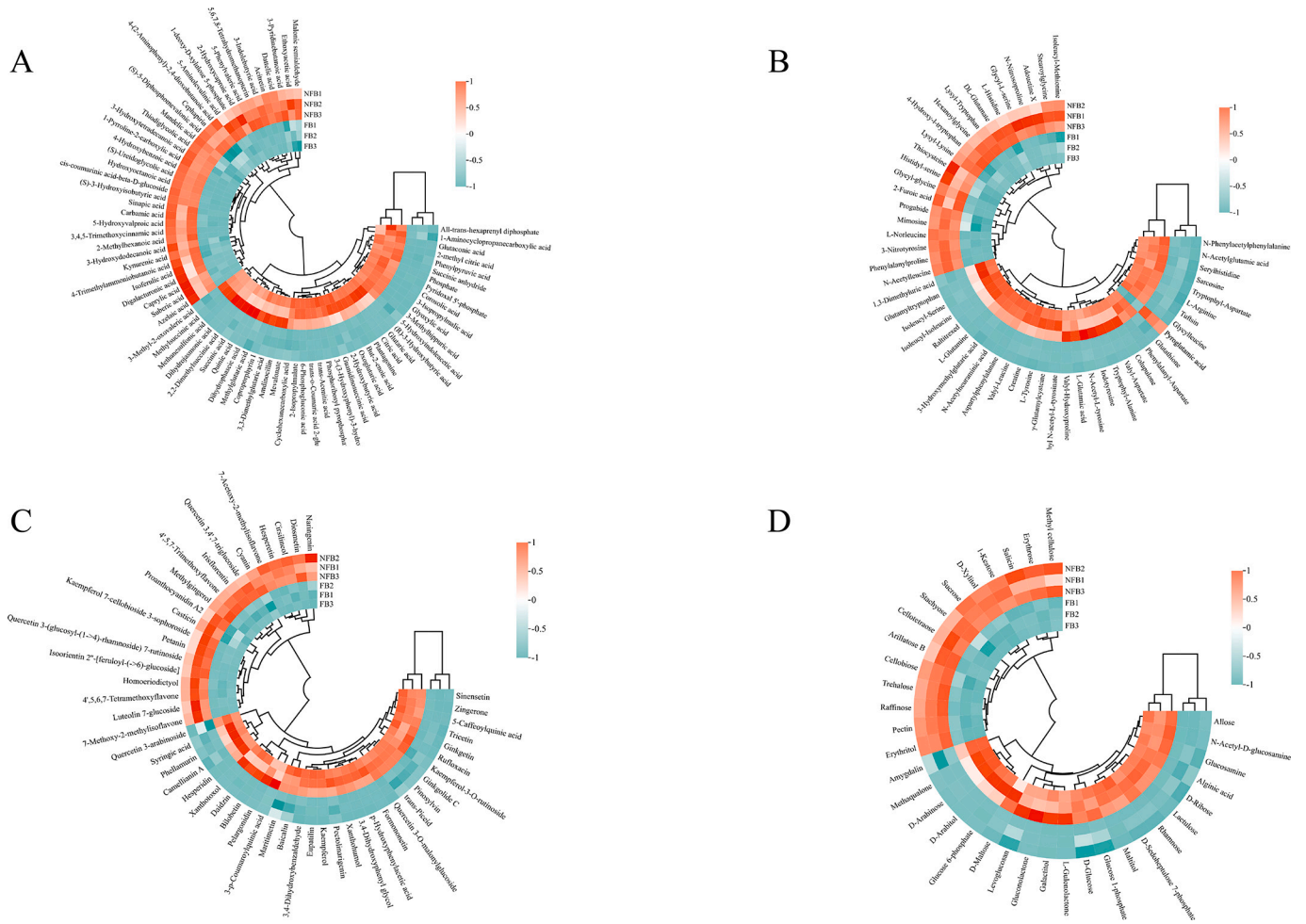


Fig. 4. Heat map of clustering of four differential metabolites. A, Organic acid and its derivatives; B, Amino acid and its metabolites; C, Flavonoids and phenolics; D, Carbohydrates and its metabolites.

Citric acid, oxoglutarate and succinic acid are the key intermediate products of the TCA cycle, which is the central metabolic pathway to produce ATP and intermediates with participates in many biosynthetic pathways. Therefore, these acid accumulations may be due to the secretion of different metabolites as a result of the large amount of material utilized by fungal growth during SSF, which act in different metabolic pathways to promote the production of organic acids (Aung, Lee, & Eun, 2022). In contrast, 27 organic acids, like 2-hydroxyoctanoic acid, octanedioic acid, azelaic acid, 2-methylhexanoic acid and indole-3-butanoic acid were down-regulated ($p < 0.01$, $Log_2FC < -2$), probably because they are intermediate products of metabolic pathways and are further converted to other metabolites.

Amino acids are also relatively active metabolites in the fermentation of *Ganoderma lucidum*, and a total of 54 amino acids and their metabolites were identified as shown in Fig. 4B. Notably, γ-glutamylcysteine in the FB sample increased by 42.39 times (in FC) compared with the NFB sample. It is a type of dipeptide containing cysteine and glutamate, and it is the precursors of glutathione (GSH) which is the tripeptide thiol with strong antioxidant activity and cellular regulatory functions (Meng et al., 2022). Therefore, γ-glutamylcysteine also possesses strong antioxidant and anti-inflammatory abilities (Braidy et al., 2019). In addition, the levels of most amino acids such as L-pyroglutamic acid, L-glutamine, L-tyrosine, L-glutamic acid, γ-glutamylcysteine and other 26 kinds of amino acids and peptides were upregulated since the extracellular enzymes produced by *Ganoderma lucidum* during SSF can degrade the crude protein in Tartary buckwheat to produce smaller molecules such as amino acids and small peptides that are more easily absorbed by the body (Zhan et al., 2023). This finding coincided with the research conducted by Zhang et al. that microbial fermentation can use protein in bee pollen to degrade macromolecules into small molecules such as peptides, small peptides and free amino acids (Zhang, Lu, & Liu, 2022). The increased amino acids and peptides not only facilitate the digestion and absorption of substances, but also change the nutritional and functional properties of substances, enhancing biological activities such as antioxidant, antibacterial and anti-inflammatory properties (Meng et al., 2022).

Phenols and ketones are the main bioactive substances in plants, in free and bound form, and are closely associated with antioxidant action (Xie et al., 2021). A total of 51 kinds of phenols and flavonoids were identified as shown in Fig. 4C. At an overall level, the content of total phenol and flavonoid increased noticeably after fermentation due to the hydrolysis of glycosidic bonds resulting from the breakdown of the microbial cell wall matrix during fermentation (Aung et al., 2022), thereby facilitating the release of phenolic compounds. Among them, 29 substances, such as quercetin 3-O-malonyl glucoside, daidzin and ginkgetin were up-regulated observably, and fermentation increased the content of hesperidin, xanthotoxol, quercetin 3-O-malonylglucoside, bilobetin and kaempferol by 240.21, 136.94, 100.77, 87.14 and 41.40 times in buckwheat, separately. However, not all ketones increased after *Ganoderma lucidum* fermentation. Particularly, 14 ketone compounds such as kaempferol 7-cellobioside 3-sophoroside, luteolin-7-O-glucoside and hesperetin were significantly down-regulated, probably because they were converted to other substances under different microorganisms and fermentation conditions or used as growth energy by fungi during

the fermentation (Xiao, Yang, et al., 2021). Many increased compounds mentioned in the present research, such as kaempferol, hesperidin, quercetin 3-O-malonylglucoside and xanthohumol, are well proven to exert potent antioxidant and anti-diabetes effects (Choi, Lee, & Lee, 2022; Katsube et al., 2006; Zhang et al., 2023; Zhu et al., 2023). Therefore, many phenolic and flavonid compounds enhanced during SSF may be one of the reasons for the increased antioxidant activity of buckwheat. *Ganoderma lucidum* fermentation also significantly affected the changes of terpenoids. Some with anti-cancer, hypoglycemic, antioxidant terpenoids and alkaloids such as arjunolic acid (Nanda, Ansari, & Khatkar, 2017) and moupinamide (Aswad et al., 2018) increased by 36.92 and 30.19 times, respectively. Beyond that, the enhancement of terpenoids and alkaloids may also be related to the health care function of fermented Tartary buckwheat.

Carbohydrate metabolism is one of the most important metabolisms during microbial fermentation. As displayed in Fig. 4D, 33 carbohydrates and their metabolites were identified, with nearly half of the sugars up-regulated and the other half down-regulated. The changes of carbohydrates and their metabolites (D-Fructose, Stachyose, Sucrose, Raffinose, Glucose, etc.) may be associated with the hydrolytic enzymes secreted by the fungus. With the increase of carbon hydrolytic enzyme activity after fermentation, the cellulose, lignin and starch in Tartary buckwheat are broken down into disaccharides and monosaccharides under the action of amylase, cellulase and xylanase. The significantly up-regulated functional sugar alcohols such as D-Arabinitol, Mannose and Erythritol are characterized with lower caloric, hypoglycemic and other biological activities (Fan, Zhang, Chen, & Cao, 2022) and can replace sucrose and glucose as an energy supplement.

3.4.3. *KEGG metabolic pathway analysis*

To further understand the corresponding metabolic pathways involved in the differential metabolites during fermentation of Tartary buckwheat by *Ganoderma lucidum*, the metabolites differentially regulated between FB and NFB were imported into the KEGG database for pathway enrichment analysis. Fig. 5 shows the 25 pathways that mainly participate in the differential metabolites. The pathways related to amino acid metabolism are mostly represented by the metabolism of amino acids, such as phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid and finally the biosynthesis of arginine; the metabolic pathways of carbohydrates include starch and sucrose metabolism as well as galactose metabolism. According to the metabolic pathway analysis in Fig. 6, sucrose can be directly produced as glucose catalyzed by sucrase-isomaltase, β-fructofuranosidase and maltase-glucoamylase, or it can be further metabolized to D-glucose-6-phosphate under the influence of sucrose synthase and enter the glycolytic pathway, causing an increase in glucose content and thus providing energy for fungal solid-state fermentation. Glucose is also involved in the pentose phosphate pathway, undergoing a series of reactions to produce D-ribose-5P, a metabolite that can be converted to D-ribose, D-sedoheptulose-7-phosphate and PRPP in purine and pyrimidine metabolism. In the galactose metabolic pathway, galactinol is hydrolyzed to produce raffinose, which is further metabolized to stachyose. After a series of hydrolysis reactions, D-mannose is produced under the hydrolysis of α-galactosidase, and then participates in amino and nucleotide sugar metabolism. The TCA cycle is a central metabolic pathway of producing ATP and intermediates, and is involved in several biosynthetic pathways. Some trichloroacetic acids, such as citric and succinic acids, act as inflammatory and cancer signals, suggesting that they play a role in regulating inflammation and cancer (Zhang et al., 2022). Aerobic bacteria release the energy contained in acetyl coenzyme A (CoA) through a sequence of processes known as the TCA cycle, which results in the oxidative synthesis of ATP and CO_2. In this metabolic pathway, citric acid, succinic acid and α-ketoglutaric acid are key components of the TCA cycle, all of which are being elevated to a great extent, probably because high glucose levels result in CoA, which increases levels of α-ketoglutaric acid while entering the TCA cycle to make citric acid. In living things, α-ketoglutaric acid participates in the TCA cycle and is a precursor in the synthesis of amino acids. It generates L-glutamate, which takes part in the metabolism of arginine, D-glutamate, and D-glutamine (Zhan et al., 2023). The significant difference in metabolites before and after fermentation results from the fact that the enzymes secreted during the fermentation of *Ganoderma lucidum* and the degree of utilization of compounds in buckwheat determine the up- and down-regulation of the final metabolites, thus influencing metabolic pathway changes.

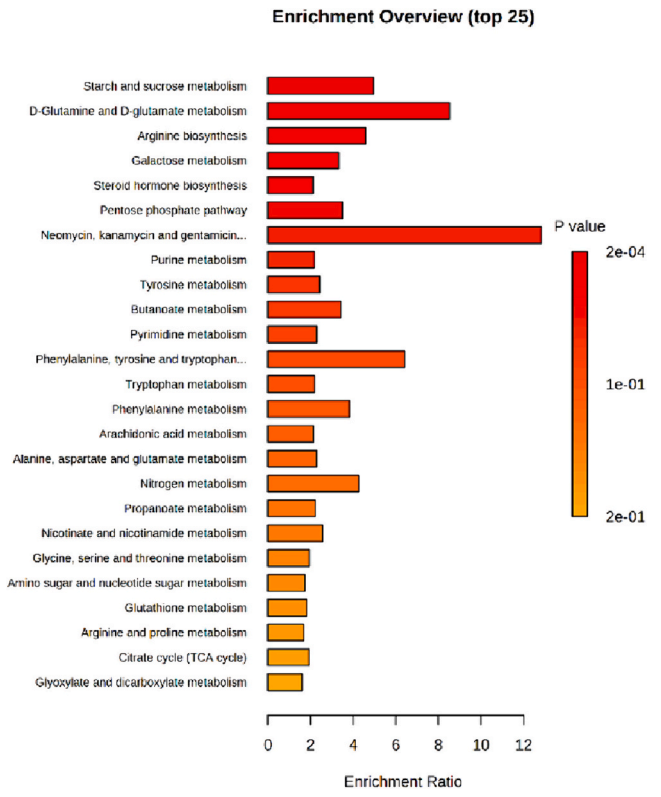


Fig. 5. Representation of the KEGG enrichment pathway.

In recent years, SSF technology has received a great deal of attention from researchers, and some LC-MS/MS has been used to investigate the metabolic pathway changes in fermentation products. Shen et al. (Shen et al., 2022) treated the pulp of sea buckthorn juice with fermentation and found that fermentation altered the metabolomics of sea buckthorn juice, remarkably enhancing flavonoids and phenolics and thus increasing antioxidant and antidiabetic effects. Based on metabolomic data, it is shown that *Ganoderma lucidum* fermentation can significantly increase the content of functional substances in Tartary buckwheat, thereby improving its antioxidant activity and anti-glycemic effects and increasing its nutritional properties. However, the preliminary metabolic profiles do not explain the underlying mechanisms for the synthesis or degradation of different metabolites and need to be further explored.

3.5. *Effect of SSF with Ganoderma lucidum on the antioxidant activity of Tartary buckwheat*

To further investigate the effect of *Ganoderma lucidum* fermentation on the antioxidant activity of Tartary buckwheat, four common antioxidant assays with different reaction mechanisms were used to evaluate the antioxidant activity of NFB and FB in this study. In Fig. 7A, it is shown that the fermentation of *Ganoderma lucidum* obviously affected the antioxidant activity of Tartary buckwheat. Consistent with the results for the active substance content, the fermentation of *Ganoderma*

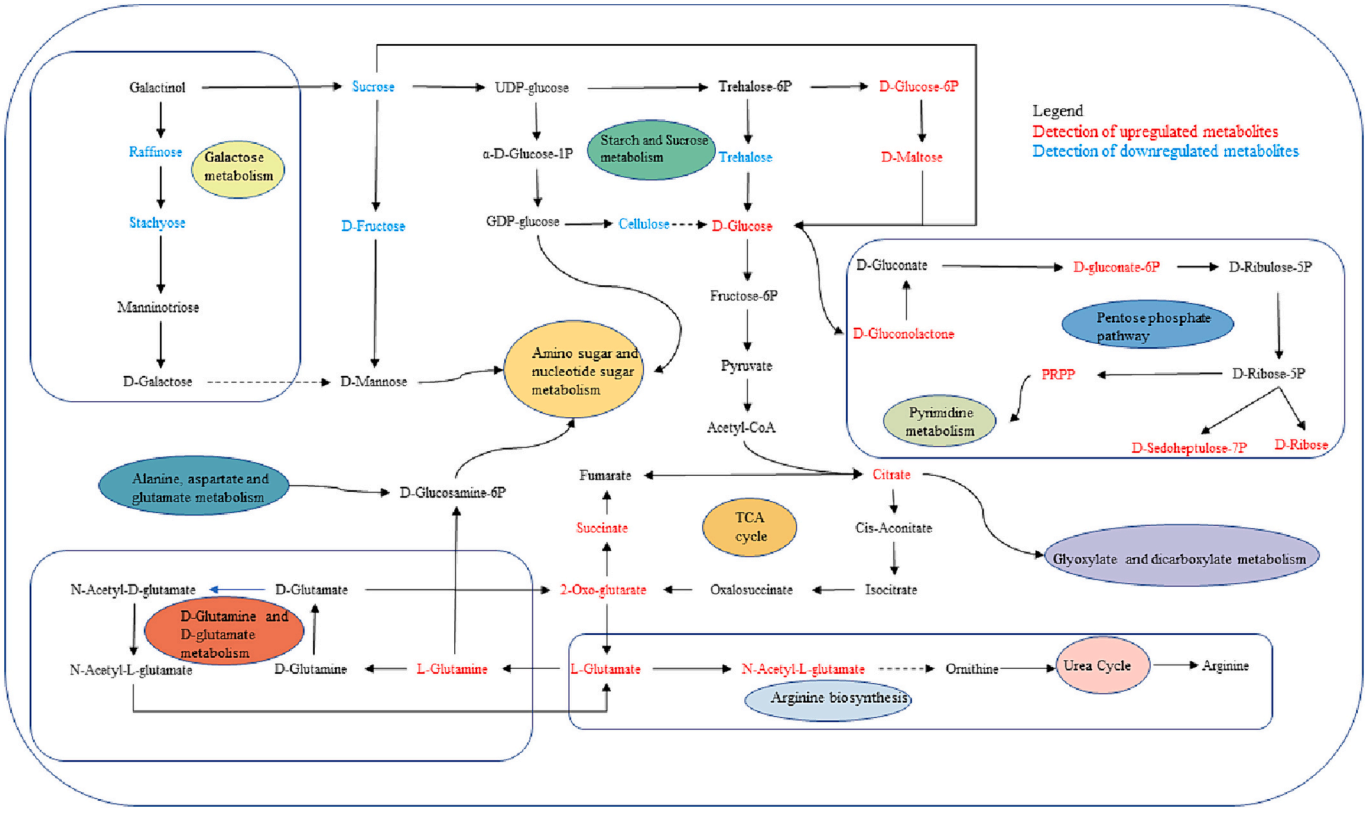


Fig. 6. Metabolic network between specific metabolites of *Ganoderma lucidum* fermented Tartary buckwheat and the KEGG pathway.

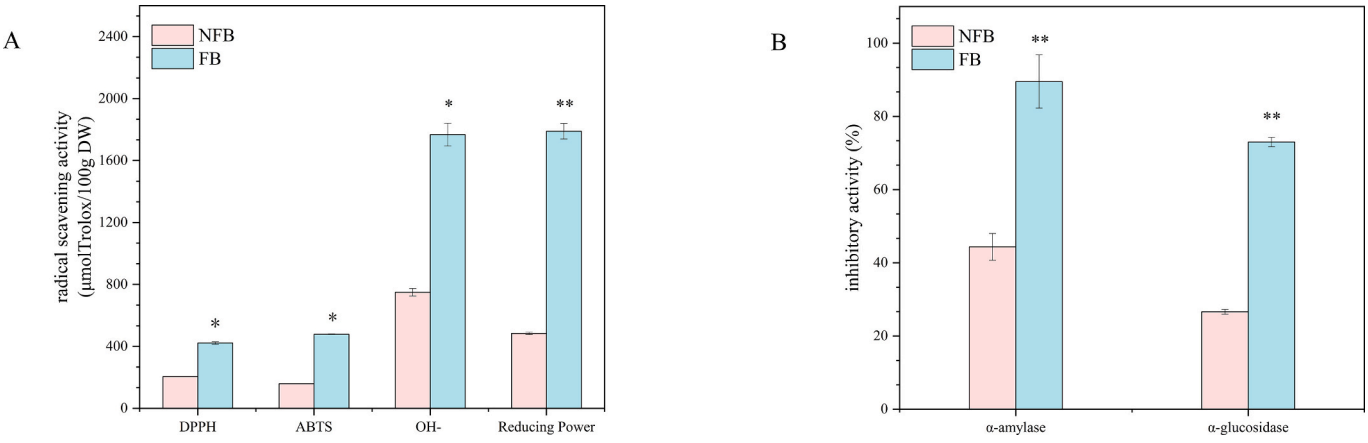


Fig. 7. A, Antioxidant activity of the FB and NFB; B, α-amylase inhibitory activity and α-glucosidase inhibitory activity of the FB and NFB.

lucidum significantly increased the antioxidant activity of Tartary buckwheat. The OH^- radical scavenging capacity, reducing power, DPPH radical scavenging capacity and $ABTS^{\cdot+}$ scavenging activity in FB were 2036.30 μmol Trolox/100 g DW, 1788.98 μmol Trolox/100 g DW, 478.60 μmol Trolox/100 g DW and 421.98 μmol Trolox/100 g DW, which are 2.28, 3.70, 2.06 and 3.00 times higher than NFB, respectively. Numerous previous studies have also demonstrated that the addition of filamentous fungi can effectively increase the antioxidant activity of fermentation products. For example, adding white truffle fungi to soybeans resulted in mycelial products with high antioxidant activity (Hsu et al., 2021). Metabolomic analysis showed that most of these compounds, such as amino acids, flavonoids, phenols, organic acids and carbohydrates, were significantly up-regulated among the main differential metabolites, while those with large differences, such as kaempferol, hesperidin, quercetin 3-O-malonyl glucoside, xanthotoxol and γ-glutamylcysteine, all possess antioxidant activity. Terpenoids, alkaloids and organic acids with antioxidant activity including moupinamide, arjunolic acid and 3-Methyl-2-oxovaleric acid were dramatically upregulated ($p < 0.01$). Therefore, the main reason for the enhanced antioxidant activity of fermented Tartary buckwheat is the fermentation process obtained during the triterpenoids, phenols, flavonoids, polysaccharides and several other bioactive substances occurring in concert.

3.6. *Effect of SSF with Ganoderma lucidum on anti-glycemic activity of Tartary buckwheat*

To explore the effect of *Ganoderma lucidum* fermentation on *in vitro* anti-glycemic activity of Tartary buckwheat, the inhibitory activity of α-amylase and α-glucosidase was elaborated. These are two key hydrolases involved in the regulation of carbohydrate metabolism *in vivo* and

affecting the glycemic index *in vivo* (Xiao, Yang, et al., 2021), because α-glucosidase inhibitors effectively affect the hydrolysis of starch, thereby reducing the release of insulin from glucose and delaying its absorption in the intestine, helping to prevent postprandial hyperglycemia. The obtained results are shown in Fig. 7B. The inhibitory activity of FB on both α-amylase and α-glucosidase was higher than that of NFB, and the α-glucosidase inhibition rate increased from 26.59% to 74.15% after fermentation treatment. In addition, α-amylase catalyzes the hydrolysis regarding the 1,4-glycosidic bonds of starch, glycogen and various oligosaccharides into simpler sugars that are readily absorbed by the intestine (Xie et al., 2021). Hence, inhibition of α-amylase in the human digestive tract is considered as one of the effective ways to control diabetes by reducing the absorption of glucose from starch (Barros et al., 2020). It was evident that the FB sample had a significant effect on the α-amylase inhibitory activity with 89.50% inhibition, a 1.02-fold increase over NFB.

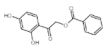
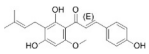
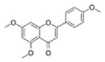
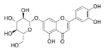
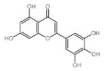
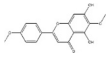
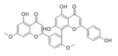
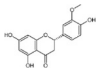
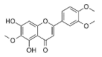
Studies have shown that phenolic and flavonoid substances have inhibitory effects on α-amylase and α-glucosidase. According to the analysis of metabolomics results, 28 of the 51 compounds screened for ketones are featured with the C6-C3-C6 basic structure of ketones, with different numbers of -OH, methoxy, isopentenyl, −glucoside and -rutinoside functional groups attached to the A and B rings (Table 3). Besides, it is also demonstrated that the number of -OH on the parent structure in flavonoids affects the inhibition of α-glucosidase and α-amylase, and in general, their inhibitory capacity increases with the number of -OH (Li et al., 2023). For example, kaempferol (with 4 -OH), xanthohumol (with 3 -OH), bilobetin (with 2 -OH), daidzin (with 1 -OH) and sinensetin (without -OH) have different numbers of -OH, so that presumably, their inhibitory activity decreases in order with the -OH group on the parent structure. Meanwhile, compared to NFB, the above compounds were upregulated to a great degree after fermentation with *Ganoderma lucidum*. Notably, kaempferol, xanthohumol and bilobetin were increased by 41.40, 70.75 and 87.14 times respectively. In addition, the substitution of -OH at the R3 position of the C ring in flavonoid compounds by glycosides or rutinosides has been shown to reduce α-glucosidase and α-amylase inhibitory activity (Han et al., 2023). Table 3 shows that the -OH at the R3 position of the C ring in quercetin 3-O-malonylglucoside, quercetin 3-arabinoside and kaempferol-3-O-rutinoside is substituted by glycosides or rutinosides, thus having a weaker inhibitory activity against α-glucosidase. However, except a 100.77-fold increase in quercetin 3-O-malonylglucoside, the other two compounds changed slightly after fermentation. The results demonstrated that the addition of *Ganoderma lucidum* fermentation enhanced the anti-glycemic effect of buckwheat.

The anti-glycemic benefits of cereals, legumes or other plant material have been reported to be enhanced by microbial fermentation (Xiao, Yang, et al., 2021), which may be related to the fermentation process, where *Ganoderma lucidum* secretes a lot of hydrolytic enzymes that release functional compounds from the Tartary buckwheat and significantly increase their content, thus achieving an anti-glycemic effect. Other than that, non-targeted metabolomics analysis showed that the fermentation of *Ganoderma* obviously increased most of the active metabolites in Tartary buckwheat, which was a key factor in improving its anti-glycemic effect. Thus, FB possesses strong α-amylase and α-glucosidase inhibitory activity and may be an important potential source of anti-glycemic and nutritional health drugs.

3.7. Pearson-related analysis

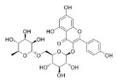
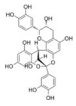
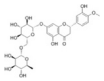
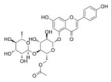
Pearson correlation coefficient analysis was performed to illustrate the correlation between bioactive substances (polysaccharides, total phenols, flavonoids, triterpenes) and antioxidant and anti-glycemic properties. As shown in Fig. 8, the four active substances were positively correlated with antioxidant and anti-glycemic activity. Total phenols and flavonoids ($r = 0.97$, $p < 0.05$) were more strongly correlated than polysaccharides (($r = 0.93$, $p < 0.05$) and triterpenes ($r =$

Table 3
Structural formulae of flavonoids. ↑ indicates that the substance has been fermented and adjusted up, ↓ indicates that the substance has been fermented and adjusted down.

Substance	Structure
Quercetin 3-O-malonylglucoside↑	
Naringenin↓	
Xanthohumol↑	
Kaempferol↑	
4′,5,7-Trimethoxyflavone↓	
Daidzin↑	
Luteolin 7-glucoside↓	
Bilobetin↑	
Diosmetin↓	
Hesperetin↓	
Tricetin↑	
Pectolinarigenin↑	
Formononetin↑	
Ginkgetin↑	
Casticin↓	
Homoeriodictyol↓	
Irisflorentin↓	
Cirsilineol↓	
Sinensetin↑	
Eupatilin↑	
Quercetin 3-arabinoside↓	

(*continued on next page*)

Table 3 (*continued*)

Substance	Structure
Kaempferol-3-O-rutinoside↑	
4′,5,6,7-Tetramethoxyflavone↓	
Baicalin↑	
Proanthocyanidin A2↓	
Hesperidin↑	
Phellamurin↑	
Camellianin A↑	

0.91, $p < 0.05$). This is similar to previous reports that antioxidant activity and anti-glycemic effects are largely dependent on specific chemical structures in flavonoids and phenolic acid substances, such as the number of aromatic and hydroxyl groups, the position of the groups and the degree of hydroxylation (Kabach et al., 2023; Yang et al., 2022). Therefore, a metabolomic analysis was carried out to correlate the substances that have been reported to have antioxidant and anti-glycemic properties. Most of the differential metabolites (including 11 flavonoids, 5 phenolic acids, 2 organic acids, 2 amino acids, 1 alkaloid, 1 terpenoid, 1 glycan and 1 nucleotide) were positively associated with antioxidant and anti-glycemic activity ($r > 0.81$, $p < 0.05$), while only 6 flavonoids, 1 alkaloid and 1 carbohydrate were negatively connected ($|r| > 0.87$, $p < 0.05$). When combined with the data analysis in Fig. 8, these differential metabolites that were positively correlated with antioxidant and anti-glycemic activity were significantly up-regulated after *Ganoderma lucidum* fermentation, while some of the down-regulated compounds were negatively correlated. This is similar to previous studies, with antioxidant activity, α-amylase and α-glucosidase inhibitory activity related to most of the differential metabolites (flavonoids, phenolic acids, terpenoids and organic acids, etc.) significantly up-regulated after SSF treatment (Chen et al., 2023; Starzyńska-Janiszewska et al., 2023; Zhao et al., 2023), and the interaction of different actives work together to promote the biological activity of Tartary buckwheat.

It has been reported that flavonoids and phenolic compounds in buckwheat are featured with antioxidant effects and potential health benefits (Xiao, Yang, et al., 2021). Besides, in this study, it is further demonstrated that the phenolics and flavonoids released by the fermentation of *Ganoderma lucidum* play a significant role in the antioxidant activity and antidiabetic activity of buckwheat.

4. Conclusion

In this study, fermentation of Tartary buckwheat with *Ganoderma lucidum* improved its antioxidant activity and anti-glycaemic capacity in an effective way. During the fermentation process, *Ganoderma lucidum* secreted a variety of hydrolytic enzymes. These extracellular enzymes degraded the cellulose on the surface of buckwheat, prompting the release of more active substances, thus enhancing the biological activity of buckwheat. A metabolomic approach using UPLC-MS/MS was taken to obtain detailed phytochemical compositions of the NFB and FB samples, and to initially explore the effect of changes in metabolites of *Ganoderma lucidum* fermented buckwheat on antioxidant activity and anti-glycaemic capacity. There were 445 compounds that differed distinctly, with most of the amino acids, organic acids, flavonoids and phenols being significantly changed after fermentation of *Ganoderma lucidum* and having strong biological activity. The results suggest that SSF using *Ganoderma lucidum* mycelia is a novel and effective means of processing and enhancing the biological activity of buckwheat. Therefore, the fermentation of Tartary buckwheat with *Ganoderma lucidum* is an excellent source of natural antioxidants, α-glucosidase and α-amylase inhibitors, which can be used to develop potential functional foods and anti-diabetic drugs.

CRediT authorship contribution statement

Rui Zhang: Writing – review & editing, Writing – original draft. **Qin Cen:** Software. **Wenkang Hu:** Investigation, Formal analysis. **Hongyan Chen:** Funding acquisition. **Fuyi Hui:** Visualization, Validation. **Jiamin Li:** Resources, Investigation. **Xuefeng Zeng:** Supervision, Methodology. **Likang Qin:** Supervision.

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.

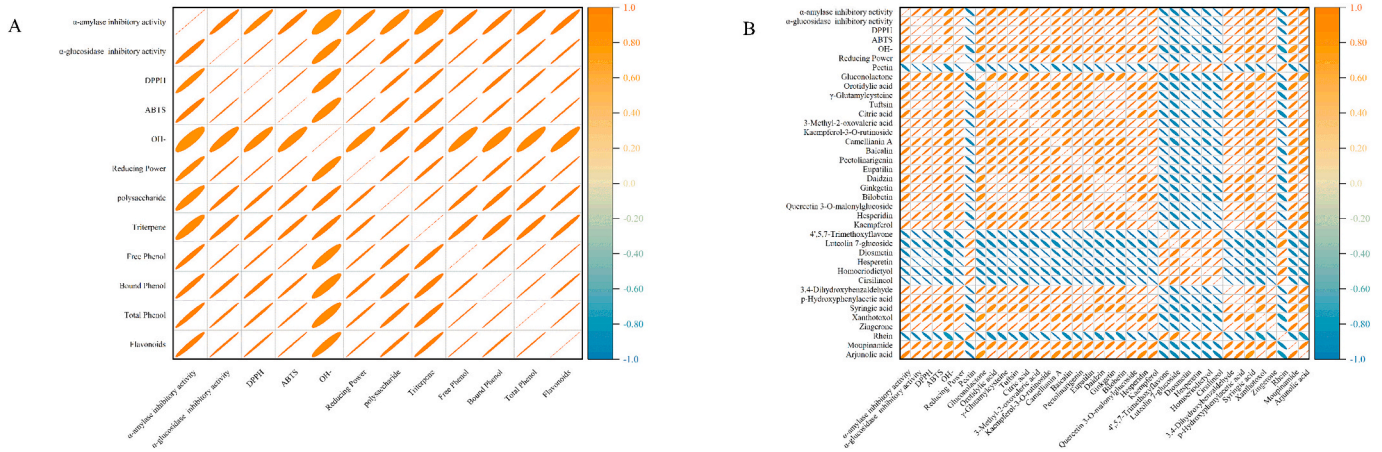


Fig. 8. Pearson's correlation coefficient analysis.

Data availability

Data will be made available on request.

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References

Almuhayawi, M. S., Hassan, A. H. A., Abdel-Mawgoud, M., Khamis, G., Selim, S., Al Jaouni, S. K., & AbdElgawad, H. (2021). Laser light as a promising approach to improve the nutritional value, antioxidant capacity and anti-inflammatory activity of flavonoid-rich buckwheat sprouts. *Food Chemistry, 345*, Article 128788. https://doi.org/10.1016/j.foodchem.2020.128788

Aswad, M., Rayan, M., Abu-Lafi, S., Falah, M., Raiyn, J., Abdallah, Z., & Rayan, A. (2018). Nature is the best source of anti-inflammatory drugs: Indexing natural products for their anti-inflammatory bioactivity. *Inflammation Research, 67*(1), 67–75. https://doi.org/10.1007/s00011-017-1096-5

Aung, T., Lee, W. H., & Eun, J. B. (2022). Metabolite profiling and pathway prediction of laver (Porphyra dentata) kombucha during fermentation at different temperatures. *Food Chemistry, 397*, Article 133636. https://doi.org/10.1016/j.foodchem.2022.133636

Barros, R. G. C., Pereira, U. C., Andrade, J. K. S., de Oliveira, C. S., Vasconcelos, S. V., & Narain, N. (2020). In vitro gastrointestinal digestion and probiotics fermentation impact on bioaccessbility of phenolics compounds and antioxidant capacity of some native and exotic fruit residues with potential antidiabetic effects. *Food Research International, 136*, Article 109614. https://doi.org/10.1016/j.foodres.2020.109614

Bei, Q., Chen, G., Lu, F., Wu, S., & Wu, Z. (2018). Enzymatic action mechanism of phenolic mobilization in oats (Avena sativa L.) during solid-state fermentation with Monascus anka. *Food Chemistry, 245*, 297–304. https://doi.org/10.1016/j.foodchem.2017.10.086

Braidy, N., Zarka, M., Jugder, B. E., Welch, J., Jayasena, T., Chan, D. K. Y., et al. (2019). The precursor to glutathione (GSH), γ-glutamylcysteine (GGC), can ameliorate oxidative damage and neuroinflammation induced by Aβ(40) oligomers in human astrocytes. *Frontiers in Aging Neuroscience, 11*, 177. https://doi.org/10.3389/fnagi.2019.00177

Chaitra, U., Abhishek, P., Sudha, M. L., Vanitha, T., & Crassina, K. (2020). Impact of millets on wheat based Belgian waffles: Quality characteristics and nutritional composition. *Lwt, 124*. https://doi.org/10.1016/j.lwt.2020.109136

Chen, Y., Liu, C., Yang, F., Chen, H., Yang, C., Fan, Z., et al. (2023). UPLC–QQQ–MS/MS-based widely targeted metabolomic analysis, antioxidant and α-glucosidase inhibitory activities of mulberry leaves processed by solid-state fermentation. *Lwt, 188*, Article 115351. https://doi.org/10.1016/j.lwt.2023.115351

Choi, S. S., Lee, S. H., & Lee, K. A. (2022). A comparative study of Hesperetin, hesperidin and hesperidin glucoside: Antioxidant, anti-inflammatory, and antibacterial activities in vitro. *Antioxidants (Basel), 11*(8). https://doi.org/10.3390/antiox11081618

Fan, Y. M., Zhang, L. H., Chen, X. Z., & Cao, Y. (2022). Metabolic modification of Candida tropicalis to produce D-arabitol from xylose. *Food and Fermentation Industries, 48*(22), 220–226. https://doi.org/10.13995/j.cnki.11-1802/ts.031153

Han, L., Wang, H., Cao, J., Li, Y., Jin, X., He, C., & Wang, M. (2023). Inhibition mechanism of alpha-glucosidase inhibitors screened from Tartary buckwheat and synergistic effect with acarbose. *Food Chemistry, 420*, Article 136102. https://doi.org/10.1016/j.foodchem.2023.136102

Hayati, S. N., Rosyida, V. T., Darsih, C., Nisa, K., Indrianingsih, A. W., Apriyana, W., & Ratih, D. (2020). Physicochemical properties, antimicrobial and antioxidant activity of ganoderma transparent soap. *IOP Conference Series: Earth and Environmental Science, 462*(1). https://doi.org/10.1088/1755-1315/462/1/012047

Heidari, F., Øverland, M., Hansen, J.Ø., Mydland, L. T., Urriola, P. E., Chen, C., et al. (2022). Solid-state fermentation of Pleurotus ostreatus to improve the nutritional profile of mechanically-fractionated canola meal. *Biochemical Engineering Journal, 187*. https://doi.org/10.1016/j.bej.2022.108591

Hsu, J. Y., Chen, M. H., Lai, Y. S., & Chen, S. D. (2021). Antioxidant profile and biosafety of white truffle mycelial products obtained by solid-state fermentation. *Molecules, 27*(1). https://doi.org/10.3390/molecules27010109

Huda, M. N., Lu, S., Jahan, T., Ding, M., Jha, R., Zhang, K., et al. (2021). Treasure from garden: Bioactive compounds of buckwheat. *Food Chemistry, 335*, Article 127653. https://doi.org/10.1016/j.foodchem.2020.127653

Kabach, I., Bouchmaa, N., Zouaoui, Z., Ennoury, A., El Asri, S., Laabar, A., et al. (2023). Phytochemical profile and antioxidant capacity, α-amylase and α-glucosidase inhibitory activities of Oxalis pes-caprae extracts in alloxan-induced diabetic mice. *Biomedicine & Pharmacotherapy, 160*, Article 114393. https://doi.org/10.1016/j.biopha.2023.114393

Katsube, T., Imawaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K., & Yamane, Y. (2006). Antioxidant flavonol glycosides in mulberry (Morus alba L.) leaves isolated based on LDL antioxidant activity. *Food Chemistry, 97*(1), 25–31. https://doi.org/10.1016/j.foodchem.2005.03.019

Li, F., Luo, T., Hou, J., Fei, T., Zhang, J., & Wang, L. (2023). Natural α-glucosidase and α-amylase inhibitors from raspberry (Rubus corchorifolius L.) leaf-tea: Screening, identification and molecular docking analysis. *Lwt, 181*, Article 114763. https://doi.org/10.1016/j.lwt.2023.114763

Li, S., Niu, H., Gao, Y., Kan, Z., Jiang, L., Li, X., & Chen, Y. (2022). Improvement of buckwheat noodles quality via differential pressure explosion puffing (DPEP): Cooking and structural quality. *International Journal of Food Engineering, 18*(6), 489–497. https://doi.org/10.1515/ijfe-2021-0290

Li, Y., Gu, F., Guo, X., Zhang, Q., Hu, R., Qin, L., et al. (2023). Effects of drying methods on bioactive components of Ganoderma lucidum fermented whole wheat in products & in vitro digestive model. *Food Research International, 168*. https://doi.org/10.1016/j.foodres.2023.112641

Lin, Y. Y., Zhao, S., Lin, X., Zhang, T., Li, C. X., Luo, X. M., & Feng, J. X. (2021). Improvement of cellulase and xylanase production in Penicillium oxalicum under solid-state fermentation by flippase recombination enzyme/ recognition target-mediated genetic engineering of transcription repressors. *Bioresource Technology, 337*, Article 125366. https://doi.org/10.1016/j.biortech.2021.125366

Lu, X., Li, F., Zhou, X., Hu, J., & Liu, P. (2022). Biomass, lignocellulolytic enzyme production and lignocellulose degradation patterns by Auricularia auricula during solid state fermentation of corn stalk residues under different pretreatments. *Food Chemistry, 384*, Article 132622. https://doi.org/10.1016/j.foodchem.2022.132622

Meng, F. B., Zhou, L., Li, J. J., Li, Y. C., Wang, M., Zou, L. H., et al. (2022). The combined effect of protein hydrolysis and lactobacillus plantarum fermentation on antioxidant activity and metabolomic profiles of quinoa beverage. *Food Research International, 157*, Article 111416. https://doi.org/10.1016/j.foodres.2022.111416

Nanda, A., Ansari, S. H., & Khatkar, S. (2017). Arjunolic acid: A promising antioxidant moiety with diverse biological applications. *Current Organic Chemistry, 21*(4), 287–293. https://doi.org/10.2174/1385272820666161017164404

Shen, F., Zhuang, J., Wang, Q., Zhang, J., Huang, Y., Mo, Q., et al. (2022). Enhancement in the metabolic profile of sea buckthorn juice via fermentation for its better efficacy on attenuating diet-induced metabolic syndrome by targeting gut microbiota. *Food Research International, 162*(Pt A), Article 111948. https://doi.org/10.1016/j.foodres.2022.111948

Starzyńska-Janiszewska, A., Stodolak, B., Duliński, R., Fernández-Fernández, C., Martín-García, B., & Gómez-Caravaca, A. M. (2023). Evaluation of saponin and phenolic profile of quinoa seeds after fungal fermentation. *Journal of Cereal Science, 111*, Article 103656. https://doi.org/10.1016/j.jcs.2023.103656

Tuly, J. A., Zabed, H. M., Nizami, A. S., Mehedi Hassan, M., Roknul Azam, S. M., Kumar Awasthi, M., et al. (2022). Bioconversion of agro-food industrial wastes into value-added peptides by a Bacillus sp. Mutant through solid-state fermentation. *Bioresour Technol, 346*, Article 126513. https://doi.org/10.1016/j.biortech.2021.126513

Wen, L., Sheng, Z., Wang, J., Jiang, Y., & Yang, B. (2022). Structure of water-soluble polysaccharides in spore of Ganoderma lucidum and their anti-inflammatory activity. *Food Chemistry, 373*(Pt A), Article 131374. https://doi.org/10.1016/j.foodchem.2021.131374

Xiao, Y., Huang, Y. X., Chen, Y. L., Fan, Z. Y., Chen, R. Y., He, C., et al. (2021). Effects of solid-state fermentation with Eurotium cristatum YL-1 on the nutritional value, total phenolics, isoflavones, antioxidant activity, and volatile organic compounds of black soybeans. *Agronomy, 11*(6). https://doi.org/10.3390/agronomy11061029

Xiao, Y., Wu, X., Yao, X., Chen, Y., Ho, C. T., He, C., et al. (2021). Metabolite profiling, antioxidant and alpha-glucosidase inhibitory activities of buckwheat processed by solid-state fermentation with Eurotium cristatum YL-1. *Food Research International, 143*, Article 110262. https://doi.org/10.1016/j.foodres.2021.110262

Xiao, Y., Yang, C., Xu, H. N., Zhang, J., & Zhang, L. F. (2021). Study on the change of flavonoid glycosides to aglycones during the process of steamed bread containing tartary buckwheat flour and antioxidant, α-glucosidase inhibitory activities evaluation in vitro. *Lwt, 145*, Article 111527. https://doi.org/10.1016/j.lwt.2021.111527

Xie, J., Liu, S., Dong, R., Xie, J., Chen, Y., Peng, G., et al. (2021). Bound polyphenols from insoluble dietary Fiber of defatted Rice bran by solid-state fermentation with Trichoderma viride: Profile, activity, and release mechanism. *Journal of Agricultural and Food Chemistry, 69*(17), 5026–5039. https://doi.org/10.1021/acs.jafc.1c00752

Yang, P., Huang, K., Zhang, Y., Li, S., Cao, H., Song, H., et al. (2022). Biotransformation of quinoa phenolic compounds with Monascus anka to enhance the antioxidant capacity and digestive enzyme inhibitory activity. *Food Bioscience, 46*, Article 101568. https://doi.org/10.1016/j.fbio.2022.101568

Yang, Z., Zeng, Y., Hu, Y., Zhou, T., Li, J., He, L., et al. (2023). Comparison of chemical property and in vitro digestion behavior of polysaccharides from Auricularia polytricha mycelium and fruit body. *Food Chem X, 17*, Article 100570. https://doi.org/10.1016/j.fochx.2023.100570

Zhan, Q., Thakur, K., Feng, J. Y., Zhu, Y. Y., Zhang, J. G., & Wei, Z. J. (2023). LC-MS based metabolomics analysis of okara fermented by Bacillus subtilis DC-15: Insights into nutritional and functional profile. *Food Chemistry, 413*, Article 135656. https://doi.org/10.1016/j.foodchem.2023.135656

Zhang, D., Ye, Y., & Tan, B. (2022). Comparative study of solid-state fermentation with different microbial strains on the bioactive compounds and microstructure of brown rice. *Food Chemistry, 397*, Article 133735. https://doi.org/10.1016/j.foodchem.2022.133735

Zhang, H., Lu, Q., & Liu, R. (2022). Widely targeted metabolomics analysis reveals the effect of fermentation on the chemical composition of bee pollen. *Food Chemistry, 375*, Article 131908. https://doi.org/10.1016/j.foodchem.2021.131908

Zhang, L. L., Li, N., Chen, S. S., Bian, X. Q., Farag, M. A., Ge, Y. H., et al. (2022). Carboxyl-containing compounds in food: Category, functions, and analysis with chemical derivatization-based LC-MS. *TrAC Trends in Analytical Chemistry, 157*. https://doi.org/10.1016/j.trac.2022.116818

Zhang, Y. F., Chen, C., Wang, N., Chen, Y., Yu, J. J., Zheng, X. C., et al. (2021). Developing a new modification technology of oat flour based on differential pressure

explosion puffing. *LWT-FOOD SCIENCE AND TECHNOLOGY, 141*. https://doi.org/10.1016/j.lwt.2021.110967

Zhang, Y. H., Zhou, J. X., Zhang, N. H., Zhao, L., Wu, W., Zhang, L. B., & Zhou, F. (2022). Process optimization for production of ferulic acid and pentosans from wheat brans by solid-state fermentation and evaluation of their antioxidant activities. *ACS Food Science & Technology, 2*(7), 1114–1122. https://doi.org/10.1021/acsfoodscitech.2c00113

Zhang, Y. Y., Zhang, P., Le, M. M., Qi, Y., Yang, Z., Hu, F. L., et al. (2023). Improving flavor of summer Keemun black tea by solid-state fermentation using Cordyceps militaris revealed by LC/MS-based metabolomics and GC/MS analysis. *Food Chemistry, 407*, Article 135172. https://doi.org/10.1016/j.foodchem.2022.135172

Zhao, Q., Yan, X., Yue, Y., Yue, T., & Yuan, Y. (2023). Improved flavonoid content in mulberry leaves by solid-state fermentation: Metabolic profile, activity, and mechanism. *Innovative Food Science & Emerging Technologies, 84*. https://doi.org/10.1016/j.ifset.2023.103308

Zhu, F. (2020). Dietary fiber polysaccharides of amaranth, buckwheat and quinoa grains: A review of chemical structure, biological functions and food uses. *Carbohydrate Polymers, 248*, Article 116819. https://doi.org/10.1016/j.carbpol.2020.116819

Zhu, L., Sun, S., Wu, W., Zhang, Y., Lin, C., & Ji, L. (2023). Xanthotoxol alleviates secondary brain injury after intracerebral hemorrhage by inhibiting microglia-mediated neuroinflammation and oxidative stress. *Neurochirurgie, 69*(3), Article 101426. https://doi.org/10.1016/j.neuchi.2023.101426