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Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>The metabolic profiling of Chinese yam fermented by *Saccharomyces boulardii* and the biological activities of its ethanol extract *in vitro*Xue Li^{a,b,1}, Yiwen Shao^{a,1}, Limin Hao^c, Qiaozhen Kang^a, Xiaolong Wang^d, Jiaqing Zhu^{a,b}, Changcheng Zhao^{a,b}, Yanling Shi^{a,b}, Jike Lu^{a,b,*}, Juanjuan Yi^{a,b,*}^a School of Life Sciences, Zhengzhou University, Zhengzhou 450001, China^b Food Laboratory of Zhongyuan, Zhengzhou University, Luohe 462300, China^c Systems Engineering Institute, Academy of Military Sciences (AMS), Beijing 100010, China^d Henan Yinfeng Biological Engineering Technology Co., Ltd., Zhengzhou 450000, China

ARTICLE INFO

Article history:

Received 10 November 2022

Received in revised form 28 January 2023

Accepted 7 March 2023

Keywords:

Chinese yam

Saccharomyces boulardii

Metabolites

Antioxidant

Immunomodulation

ABSTRACT

Chinese yam (*Dioscorea opposita* Thunb.), as one of the medicinal and edible homologous plants, is rich in various nutrients and functional factors. In this study, Chinese yam fermented by *Saccharomyces boulardii* was performed to investigate its bioactive components and metabolic profile. And then, the main bioactive components and biological activities of fermented Chinese yam ethanol extract (FCYE) were evaluated. Results showed that there were 49 up-regulated metabolites and 52 down-regulated metabolites in fermented Chinese yam compared to unfermented Chinese yam. Besides, corresponding metabolic pathways analysis initially revealed that the distribution of bioactive substances was concentrated on alcohol-soluble small molecular substances. Ulteriorly, the total polyphenol content and the total flavonoid content in FCYE were significantly increased, and the corresponding antioxidant and immunomodulatory activities *in vitro* were also significantly enhanced. Our study provided a new reference for the comprehensive utilization of Chinese yam and laid a theoretical foundation for the development and application of natural probiotic-fermented products.

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1. Introduction

Chinese yam (*Dioscorea opposita* Thunb.), also known as Huai yam, is a traditional food and medicine in China, mainly growing in Jiaozuo City, Henan Province. It is rich in nutrients and functional components, such as starch, polysaccharides, proteins, polyphenols, flavonoids, etc.^[1], along with various biological activities including antidiabetic, antioxidant, immunomodulatory activities and so on^[2]. The inherent nutritional factors and bioactive components of Chinese

yam make it possess important application value in the food field of human nutrition and health. At the same time, with the progress of society and people's living quality, functional foods beneficial to human health have attracted more and more attention. Recently, Chinese yam has become a research focus and the development and processing industry of related products have gradually developed. Combined with the traditional technology of microbial fermentation, previous study has developed some potentially healthy products such as yam wine, yam bread and yam yogurt^[3]. Additionally, anti-nutritional compounds including oxalate and phytate in yam fermented by *Leuconostoc lactis* CCMA 0415 could be significantly reduced^[4]. Noteworthy, the ethanol extract of *Monascus*-fermented *Dioscorea* could enhance significantly the oxidative stress resistance via DAF-16/FOXO in *Caenorhabditis elegans*^[5]. And breads made

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of wheat flour substituted with no more than 15% Chinese yam presented a high quality and better antioxidant properties^[6]. However, it was still desperately needed for more and further exploration of deep-processed products for Chinese yam.

With more consumer awareness toward healthy diet, the development of probiotics-fermented functional foods was researched increasingly. *Saccharomyces boulardii*, as a probiotic yeast, displays favorable bioactivities including antioxidant, immunomodulatory, intestinal probiotic effects and so on^[7]. Recently, in the field of food fermentation, its application potential has been gradually explored. For example, *S. boulardii* could be well combined into dairy-based food products to progress functional and therapeutic nutrients. A synbiotic yogurt by incorporation of *S. boulardii* and inulin improved its textural and sensory properties, as well as exerting the beneficial probiotic effects and increasing by 12 volatile compounds. Additionally, during the beer production process, the utilization of *S. boulardii* allowed breweries to offer innovative low-ethanol or non-ethanol products to customers, along with improved flavor and health characteristics containing stronger antioxidant activity and higher yeast viability^[8]. Also, the probiotic yeast provided such abilities as improving nutritional value, digestion of food ingredients and wholesome bioactivities in the fermentation of fruit and vegetable cereal products. The black carrot juices fermented by *S. boulardii* exhibited high antioxidant activity that was associated with increased concentrations of total polyphenol compounds, as well as a decent biological accessibility^[9]. In view of the probiotic and fermentative properties possessed by *S. boulardii*, its fermentation products exists a promising potential to be tapped in the field of fermented functional foods.

Further metabolic profiling and related pathways analysis of products from probiotic fermentation are notably meaningful for the development of functional fermented foods. For example, the probiotic-fermented unhopped beer (yeast and lactic acid bacteria) showed enriched derivatives of amino acids (e.g., tryptophan metabolites) and organic acids (e.g., indoleacetic acid), indicating excellent antioxidant activity^[10]. Additionally, the fermentation of ginkgo rice wine conducted to synthesize ginkgolide C and degrade ginkgolic acid biologically and the metabolites correlated closely with its quality^[11]. The above studies indicated that the differences in metabolites significantly affected the quality and biological activities of the fermentation products. Therefore, the preliminary study on the metabolic characteristics of fermented foods contributes to the investigation of the metabolic substances changes in the fermentation, and provides a certain theoretical basis for the development of functional foods.

To our knowledge, it barely has reports about Chinese yam fermentation by *S. boulardii*. In fact, the acceptable feasibility of fermented Chinese yam products using *S. boulardii* was investigated in our previous study, where the fermented polysaccharides showed better functional properties including antioxidant and radioprotection effects^[12]. However, metabolic profiling and bioactivities are not yet studied to understand the metabolites differences and functional changes of probiotic-fermented yam. Therefore, this study aimed to explore differential bioactive metabolites and related metabolic pathways during Chinese yam fermentation and to determine the potential bioactivities of fermented Chinese yam ethanol extract (FCYE) for its processing and development in functional food industry (Fig. 1).

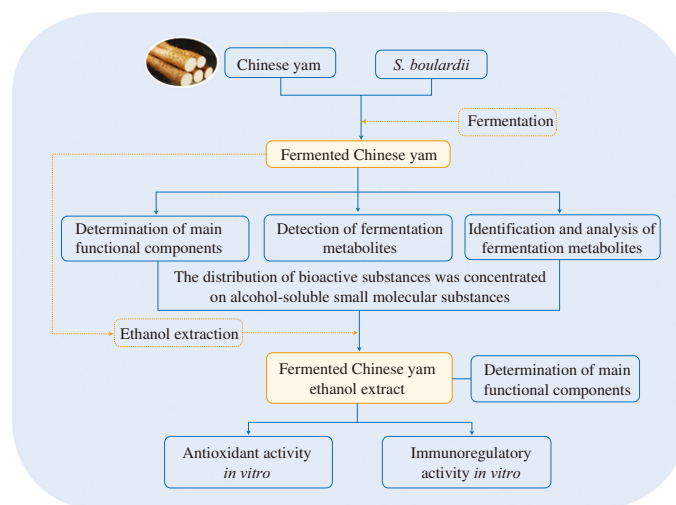


Fig. 1 Scheme of the experiments.

2. Materials and methods

2.1 Materials and chemicals

Chinese yam (*D. opposita* Thunb.) was bought from Jiaozuo City (Henan, China). The strain (*S. boulardii*) was obtained from Tianjin University of Science and Technology. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Cell Counting Kit-8 (CCK-8) was bought from Topscience Co., Ltd. (Shanghai, China). Nitric Oxide Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). All other reagents and chemicals used in this study were of analytical grade.

2.2 Fermentation of Chinese yam

The *S. boulardii* starter culture and fermentation of Chinese yam were performed in accordance to the previous method with slightly modifications^[12]. Briefly, the fresh yams were washed, sliced, dried at 45 °C. Yam powder was ground using a small swing-type crushing machine (Yongkang Hongsun Electromechanical Co., Ltd., Henan, China) and sifted with 40-mesh. The constituent ratio of the yam medium was an appropriate proportion of yam powder (4%) and peptones (1%). The pH was adjusted to 5.50. The sterilized yam mediums (sterilized at 121 °C for 20 min) were fermented at 2% (V/V, final concentration, approximately 8 lg (CFU/mL)) at 180 r/min and 30 °C for 24 h. Fermented Chinese yam (FCY) was freeze-dried and preserved at −20 °C. The control group (unfermented Chinese yam, UCY) was obtained as the same steps except for 0 inoculum.

2.3 Determination of main functional components

The total sugar contents were determined by the phenol-sulfuric acid method based on the previous study. Determination of protein contents in samples were performed by coomassie brilliant blue method. The total polyphenol content (TPC) and the total flavonoid

content (TFC) were analyzed respectively by the Folin-Ciocalteu colorimetric method and $\text{NaNO}_2\text{-AlCl}_3\text{-NaOH}$ method^[13].

2.4 Detection of fermentation metabolites

The freeze-dried UCY and FCY were dissolved in 70% methanol solution and extracted at 4 °C overnight, respectively. And then both extracts were severally centrifuged (12 000 r/min, 10 min) to obtain two kinds of supernatant for following analysis^[14].

Ultra-high performance liquid chromatography (UPLC) was based on the Shimadzu Nexera X2 system equipped with an Agilent SB-C₁₈ (2.10 mm × 100 mm, 1.80 μm). The volume injection was 4 μL and the programmed temperature was 40 °C. Additionally, the mobile phase consisted of water (A) and acetonitrile (B) (both contained 0.10% formic acid) with a flow rate of 0.35 mL/min. The gradient elution procedure was as follows: 0–9 min, 5%–95% B; 9–10 min, 95% B; 10.00–11.10 min, 95%–5% B; and 11.10–14.00 min, 5% B.

Tandem mass spectrometry (MS/MS) analysis was performed with an AB Sciex 4500 QTRAP system (Thermo Fisher, MA, USA) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization modes controlled by Analyst 1.6.3 software (AB Sciex). The ESI parameters were set as follows: capillary temperature, 550 °C; spray voltage, 5 500 V (positive)/–4 500 V (negative); gas I, 50 psi; gas II, 60 psi; curtain gas, 25 psi; mass scan range, m/z 100–1 250. The mode of triple quadrupole mass spectrometry was the multiple reaction monitoring (MRM) mode. The tuning and mass calibration was performed using polypropylene glycol solution.

2.5 Identification and analysis of fermentation metabolites

Based on Kyoto Encyclopedia of Genes and Genomes (KEGG), Yeast Metabolome Database (YMDB) and a Metware Database (MWDB), the fermentation metabolites were identified and analyzed according to the Metabolomics Standards Initiative (MSI).

2.6 Preparation of FCYE

FCYE was obtained by ethanol extraction method^[15]. The freeze-dried powder of FCY was mixed with 60% ethanol with the ratio of solid to liquid at 1:30 (g/mL) and the mixture was extracted in a 60 °C water bath for 60 min. Then vacuum filtration was performed to acquire the extracting solution and the precipitate was extracted again by the same method. Subsequently, the filtered solution was concentrated by the rotary vacuum evaporator and it was freeze-dried and stored at –20 °C for following assays. The unfermented Chinese yam ethanol extract (UCYE) was obtained in a similar way except that UCY was the extraction material. TPC and TFC in FCYE were detected by the Folin-Ciocalteu colorimetric method and the $\text{NaNO}_2\text{-AlCl}_3\text{-NaOH}$ method, respectively.

2.7 Determination of biological activities of FCYE

The bioactivities of FCYE were evaluated and compared with UCYE on the antioxidant and immunomodulatory activities.

2.8 Determination of biological activities of FCYE

2.8.1 Antioxidant activity

Total reducing power (TRP), DPPH and ABTS scavenging activities of FCYE were respectively determined in accordance with our previous methods with some modifications^[15].

2.8.2 Immunomodulatory activities

The *in vitro* immunomodulatory activity of FCYE was performed on RAW264.7 macrophages, which were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in a cell incubator (37 °C, 5% CO₂) using DMEM high-glucose medium containing 10% fetal bovine serum. The effects of FCYE on cell viability, phagocytosis activity and NO production ability were evaluated by CCK-8 method, neutral red method and nitric oxide assay kit, respectively^[16].

2.9 Statistical analysis

Data were processed by the GraphPad Prism 8.0 software and significant differences between two groups were determined by Student's *t*-test, while the ones among three or more groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Results were expressed as means ± SD from three parallel experiments. Statistical significance was designated as **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, respectively.

3. Results and discussion

3.1 Analysis of main functional components of FCY and UCY

In order to explore the effect of fermentation by *S. boulardii* on the main functional components in Chinese yam, the total sugar, protein, TPC and TFC were determined and analyzed (Table 1). Compared with UCY, the total sugar contents in FCY were significantly decreased (*P* < 0.05), which were mainly attributed to the fact that *S. boulardii* could metabolize hexoses to monosaccharides or disaccharides as its main carbon source when growing in the saccharide-rich substrate of Chinese yam, with the production of CO₂, H₂O and ethanol^[7]. There were no significant difference in protein contents between UCY and FCY (*P* > 0.05). However, it was worth noting that the TFC after fermentation increased obviously from (120.90 ± 1.56) mg RE/100 g to (131.71 ± 0.96) mg RE/100 g, the differences in specific fermentation metabolites were further investigated by the UPLC-MS/MS method.

Table 1
Main functional components of FCY and UCY.

Components	UCY	FCY
Total sugar (mg/g DW)	705.10 ± 12.12	607.98 ± 22.65**
Protein (mg/g DW)	16.49 ± 0.12	16.96 ± 0.51
TPC (mg GAE/100 g DW)	435.82 ± 9.80	416.03 ± 4.85*
TFC (mg RE/100 g DW)	120.90 ± 1.56	131.71 ± 0.96***

Note: Data were expressed as mean ± SD (*n* = 3). Significant difference from UCY was designated as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, respectively.

3.2 Analysis of differential metabolites in FCY and UCY

Based on the determination of main functional components in UCY and FCY, UPLC-MS/MS was utilized to further investigate the metabolites differences before and after fermentation. The FCY components were further analyzed by mass spectrometry based on MWDB database. Compared with UCY, a total of 101 different metabolites in FCY were screened according to the differential fold change (FC) ($FC > 2$ or $FC < 0.50$). Among the identified differential metabolites, 49 metabolites were significantly up-regulated which mainly concentrated in organic acids, flavonoids and nucleotide derivatives. Another 52 metabolites were observably down-regulated, which mainly concentrated in sugars, lipids, nucleotides and amino acids. As shown in Table 2, the top 30 differential metabolites were screened according to the fold change and normalized abundance. Consistent with these results, previous research showed that the majority of identified metabolites in ginkgo rice wine fermented by *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* were organic acids and derivatives and organic oxygen-containing compounds^[11]. Simultaneously, similar metabolites including flavonoids and phenolic acids existed in vegetable juice fermented with *S. cerevisiae* and *S. boulardii*^[9].

Table 2
Relative levels differences of main metabolites in FCY and UCY.

Compounds	Formula	RT (min)	M _w (Da)	Type	Fold change (FCY/UCF)
D-Fructose	C ₆ H ₁₂ O ₆	0.76	180.06	Down	0.092 1
D-Glucose	C ₆ H ₁₂ O ₆	0.82	180.06	Down	0.085 8
D-Sucrose	C ₁₂ H ₂₂ O ₁₁	0.78	342.12	Down	0.139 0
D-Glucuronic acid	C ₆ H ₁₀ O ₇	0.72	194.04	Up	3.766 8
N-Glycyl-L-leucine	C ₈ H ₁₆ N ₂ O ₃	1.92	188.12	Down	0.025 8
L-Glutamic acid	C ₅ H ₉ NO ₄	0.83	147.05	Down	0.354 8
3-Hydroxy-3-methylpentane-1,5-dioic acid	C ₆ H ₁₀ O ₅	1.40	162.05	Up	8.719 2
L-Aspartic acid	C ₄ H ₇ NO ₄	0.71	133.04	Down	0.164 1
Glutathione (GSH)	C ₁₀ H ₁₇ N ₃ O ₆ S	1.14	307.08	Up	135.380 0
10-Heptadecenoic acid	C ₁₇ H ₃₂ O ₂	11.47	268.24	Up	3.720 6
Tryptophol	C ₁₀ H ₁₁ NO	4.98	161.08	Up	21.825 0
L-Glycyl-L-phenylalanine	C ₁₁ H ₁₄ N ₂ O ₃	2.26	222.10	Down	0.027 6
α -Linolenic Acid	C ₁₈ H ₃₀ O ₂	10.66	278.23	Down	0.288 3
Riboflavin (vitamin B ₂)	C ₁₇ H ₂₀ N ₄ O ₆	3.21	376.14	Up	3.724 1
2-Hydroxyisocaproic acid	C ₆ H ₁₂ O ₃	3.51	132.08	Up	18.645 0
DL-3-Phenyllactic acid	C ₉ H ₁₀ O ₃	3.85	166.06	Up	18.996 0
Allantoin	C ₄ H ₆ N ₄ O ₃	0.90	158.04	Up	2.535 7
Phenylpyruvic acid	C ₉ H ₈ O ₃	3.71	164.05	Up	13.238 0
2-Isopropylmalic acid	C ₇ H ₁₂ O ₅	2.74	176.07	Up	2.191 3
Methylenesuccinic acid	C ₅ H ₆ O ₄	0.86	130.03	Up	2.346 2
3,7-Di-O-methylquercetin	C ₁₇ H ₁₄ O ₇	6.32	330.07	Up	7.916 7
Pinobanksin	C ₁₅ H ₁₂ O ₅	5.45	272.07	Up	2.269 6
Butin	C ₁₅ H ₁₂ O ₅	5.60	272.07	Up	2.309 9
Galangin	C ₁₅ H ₁₀ O ₅	5.66	270.05	Up	2.035 7
2-Hydroxyadenosine	C ₁₀ H ₁₃ N ₅ O ₅	1.81	283.09	Up	2.307 6
2'-Deoxyadenosine	C ₁₀ H ₁₃ N ₅ O ₃	1.96	251.10	Up	2.522 2
2'-O-Methyladenosine	C ₁₁ H ₁₅ N ₅ O ₄	2.08	281.11	Up	6.019 2
2'-Deoxycytidine	C ₈ H ₁₃ N ₃ O ₄	1.13	227.09	Up	2.096 4
2,6-Dimethoxybenzaldehyde	C ₉ H ₁₀ O ₃	3.97	166.06	Up	17.566 9
2-Hydroxy-3-phenylpropionic acid	C ₉ H ₁₀ O ₃	4.12	166.06	Up	19.924 9

Note: This table listed the top 30 metabolites according to the metabolic differences and normalized abundance.

3.3 Metabolic profile analysis in FCY

During microbial fermentation, different metabolites cooperate with each other to produce bioactive compounds or perform biological activities^[17]. Based on related metabolic pathways derived from the enrichment of differential metabolites, the metabolic profile in FCY was ulteriorly analyzed, which was beneficial to explore the potential bioactivities of fermented products and the interaction of different metabolites. The main metabolic pathways in FCY were shown in Fig. 2A, where it was found that there were 4 metabolic pathways with the most significant changes, namely riboflavin metabolism pathway, α -linolenic acid metabolism pathway, galactose metabolism pathway and zeatin biosynthesis pathway. Additionally, the fermentation also caused other metabolite pathways changes including starch and sucrose metabolism pathway, flavonoid biosynthesis pathway, pyruvate metabolism pathway, amino sugar and nucleoside sugar metabolism pathway, nicotinate and nicotinamide pathway.

As shown in Fig. 2B, the metabolic network in FCY was summarized according to the metabolic pathways and differential metabolites. The starch in Chinese yam could be hydrolyzed into glucose through starch and sucrose metabolism pathway and glycolysis pathway. Meanwhile, metabolites (phosphoenolpyruvate and pyruvate) entered the tricarboxylic acid (TCA) cycle and C5-branched dibasic acid metabolism pathway to further produce numerous metabolites including organic acid (e.g. itaconate, α -ketoglutaric acid, etc.). In the galactose metabolism pathway, galactokinase and α -D-galactose-1-phosphate uridylyltransferase could convert galactose into α -D-glucose-1-phosphate, which probably further resulted in a significant increase in the contents of D-galacturonic acid and D-glucuronic acid by pentose and glucuronate interconversions pathway.

There were abundant essential amino acids in Chinese yam such as glutamate, leucine, phenylalanine and so on^[18]. Most of aldehydes, alcohols, esters and ketoacids were derived and transformed from amino acids in the process of microbial metabolism. Also, the amino acid transamination reaction contributed to forming the flavor of fermentation products^[19]. During the fermentation of Chinese yam by *S. boulardii*, it was found that five amino acid metabolism pathways showed significant changes based on the differences of metabolites, involving leucine, tryptophan, glutamate, lysine and phenylalanine. For example, the phenylalanine could generate phenylpyruvate by amino acid oxidase and produce phenyllactate and 2-hydroxy-3-phenylpropionic acid through phenylalanine metabolism pathway, along with ulteriorly *p*-coumaryl alcohol being converted by phenylpropanoid biosynthesis pathway. Generally, the amino acid derivatives produced by *S. boulardii* fermentation chiefly concentrated on organic acids and phenolic acids, which were mostly related to the antioxidant and antibacterial activities, such as DL-3-phenyllactic acid, 2-hydroxyisocaproic acid, 2-hydroxy-3-phenylpropionic acid and so on^[20-21]. In addition, both phenylalanine and glutamate also possessed the characteristic that improved food flavor and enriched various flavor substances produced by reacting with amino sugars^[22].

Previous research has shown that the polyphenols accounted for one of active ingredients in Chinese yam, which displayed favorable bioactivities such as antioxidant, antidiabetic, hypotensive activities and so on^[23]. It was presumed that the significant increase of TFC in FCY was mainly due to the formation of flavonoids (like

pinobanksin, galangin, butin, etc.) from *p*-coumaric acid through the phenylpropanoid biosynthesis pathway and the flavonoid biosynthesis pathway. At the same time, the naringenin could be converted respectively into eriodictyol, apigenin and dihydrokaempferol under the action of flavonoid hydroxylase, synthase and dioxygenase. Besides, 3,7-*O*-dimethylquercetin might be obtained from quercetin through flavone and flavonol biosynthesis pathways. Emphatically, the study has proved that flavonoids correlated closely with the antioxidant activity, which could be used as one of representative indicators for evaluating the antioxidant activity of natural or processed products^[24]. Otherwise, it was worth noting that flavonoids (such as quercetin, apigenin, etc.) also exhibited significant immunomodulatory potential in both specific and non-specific immunomodulation^[25].

Fatty acids is one of the common nutrients in Chinese yam, mainly containing oleic acid, linoleic acid and linolenic acid^[26]. The reduction of α -linolenic acid and the increase of traumatic acid during the fermentation might be relevant to the α -linolenic acid metabolism pathway. Notably, the traumatic acid biosynthesis might start either from linoleic acid or linolenic acid, which were both 18-carbon unsaturated fatty acids precursors. Meanwhile, traumatic acid, with the similar bioactivity to unsaturated fatty acids, also showed good antioxidant activity^[27].

Adenosine and allantoin were both representative secondary metabolites of Chinese yam, with a remarkable increase in differential fermented metabolites, which were probably processed by purine metabolism pathway and zeatin biosynthesis pathway. Furthermore, fermentation also promoted an apparent increase of riboflavin (vitamin B₂) and ribitol, which was completed by guanosine 5'-monophosphate via the riboflavin metabolism pathway. It was also consistent with a previous study where the incorporation of *Lactococcus lactis* N8 and *S. boulardii* SAA655 could significantly enhance the contents of B-vitamins (riboflavin and folate) in idli batter^[28], which seemed to indicate that *S. boulardii* might be a potential source capable of synthesizing B-vitamins. The reduction of NADP⁺, NAD⁺ and nicotinate *D*-ribonucleoside during the fermentation might be relevant to the nicotinate and nicotinamide metabolism pathway. Nicotinamide

was a vitamin B₃ precursors of NAD in the human diet, and NAD was required for cellular bioenergetics and metabolism and was also a critical regulator of key cellular processes^[29].

Based on the composition and metabolism analysis of FCY, it initially indicated that the following extraction of bioactive compounds should focus on alcohol-soluble small molecules and the *in vitro* biological activities of such extract were explored subsequently.

3.4 Analysis of main functional components of FCYE and UCYE

Polyphenol compounds are the main bioactive components of ethanol extract of Chinese yam and have strong antioxidant activity^[30]. The results in Table 3 showed that the TPC and TFC of FCYE were markedly improved after fermentation. Compared with UCYE, the TPC and TFC were significantly increased by 14.29% ($P < 0.001$) and 44.37% ($P < 0.001$), respectively. Previous studies have shown that polyphenol compounds could be biotransformed by microorganisms during fermentation^[31-33]. In particular, the glutinous rice, puerariae radix and other functional foods had an increased TPC and enhanced antioxidant activities after fermentation^[32-33].

Table 3
Main functional components of FCYE and UCYE.

Component	UCYE	FCYE
TPC (mg GAE/100 g DW)	674.16 ± 12.38	770.53 ± 9.85***
TFC (mg RE/100 g DW)	294.13 ± 6.06	424.64 ± 5.17***

Note: Data were expressed as mean ± SD ($n = 3$). Significant difference from UCYE was designated as *** $P < 0.001$.

3.5 Antioxidant activities of FCYE

The antioxidant activity of functional food was the basis for its various effects and it was also one of the functional properties that consumers paid attention to. The *in vitro* antioxidant activities of FCYE were evaluated and shown in Fig. 3. It could be seen from Fig. 3A that TRP of FCYE gradually increased and exerted better

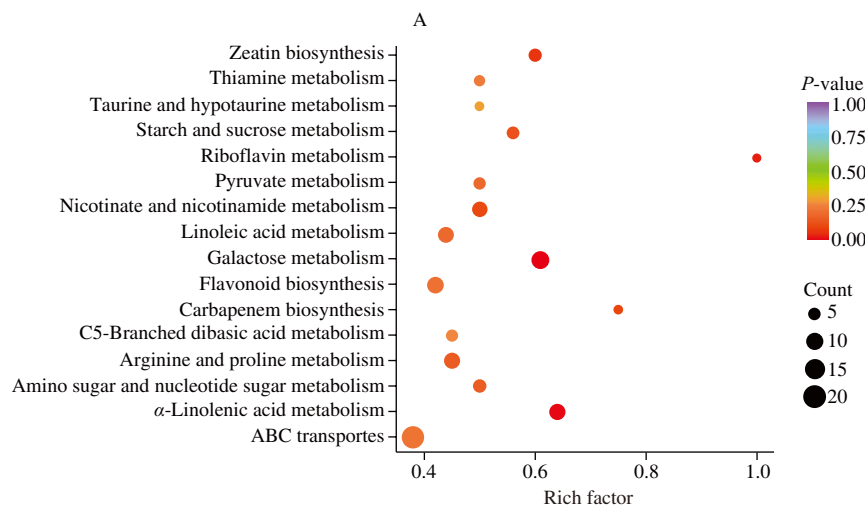


Fig. 2 Metabolic profiling of Chinese yam fermented by *S. boulardii*. (A) Main metabolic pathways during the fermentation; (B) Analysis of the metabolic network during the fermentation of Chinese yam by *S. boulardii*. Significantly up-regulated substances were shown in red and significantly down-regulated substances were shown in blue.

B

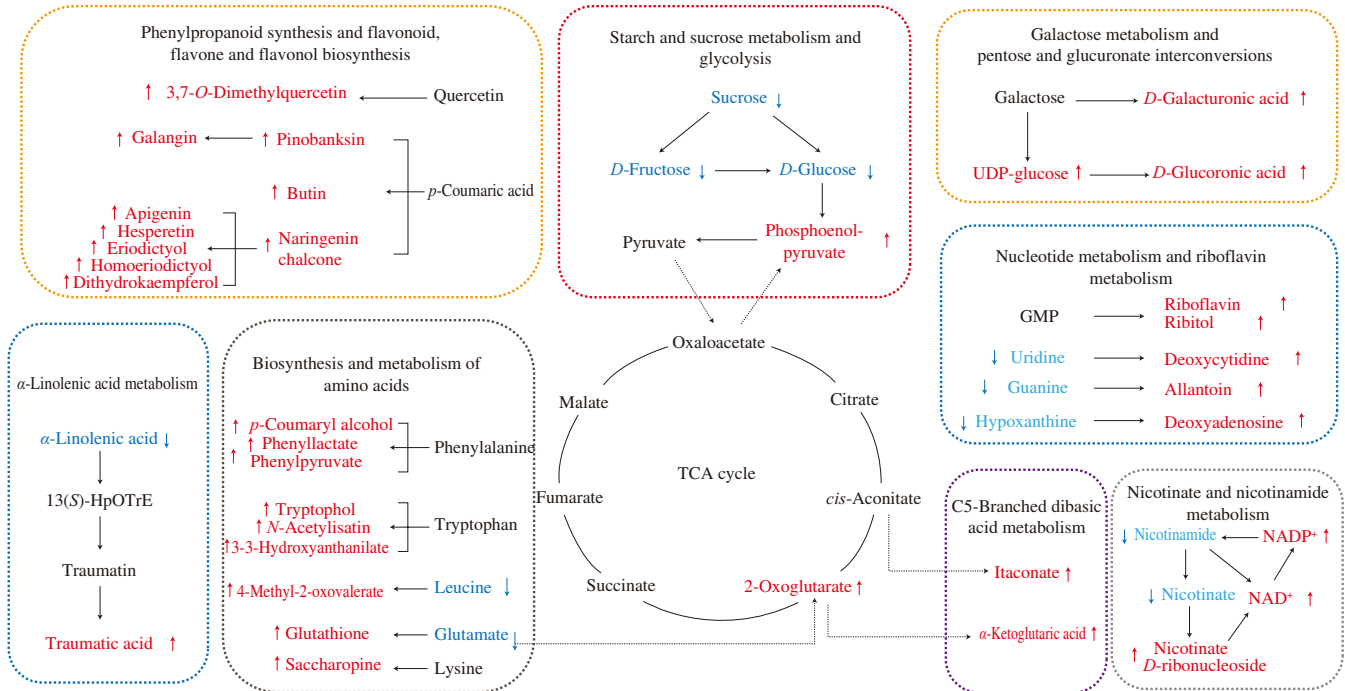


Fig. 2 (Continued)

than UCYE in the concentration range of 1.00–5.00 mg/mL, which might be attributed to the higher proportion of flavonoids, phenolic acids, organic acids and other reducing small molecules in FCYE. Notably, DPPH is a kind of stable organic nitrogen free radical and its determination is based on the electron transfer reaction^[34]. In Fig. 3B, DPPH scavenging activity of FCYE increased in a dose-dependent manner with half maximal inhibitory concentration (IC_{50}) values of (0.95 ± 0.03) mg/mL, while IC_{50} values of UCYE was (1.34 ± 0.02) mg/mL. It was obvious that FCYE possessed stronger DPPH free radical scavenging ability ($P < 0.001$). As shown in Fig. 3C, FCYE also displayed a dose-effect relationship with the scavenging ability of ABTS

free radicals. The calculated IC_{50} value of FCYE ((4.36 ± 0.10) mg/mL) was significantly lower than that of UCYE ((5.51 ± 0.13) mg/mL) ($P < 0.001$), which showed consistency with their DPPH scavenging activities. Interestingly, ABTS is a non-physiological free radical generated by persulfate oxidation and its reduction in the presence of antioxidants is related to both the hydrogen atom transfer mechanism and the electron transfer reaction^[35]. Therefore, the higher ABTS scavenging activity of FCYE was probably ascribed to not only the fermented metabolites of *S. boulardii* but also the production of more free hydroxyl groups caused by the degradation of macromolecules during fermentation.

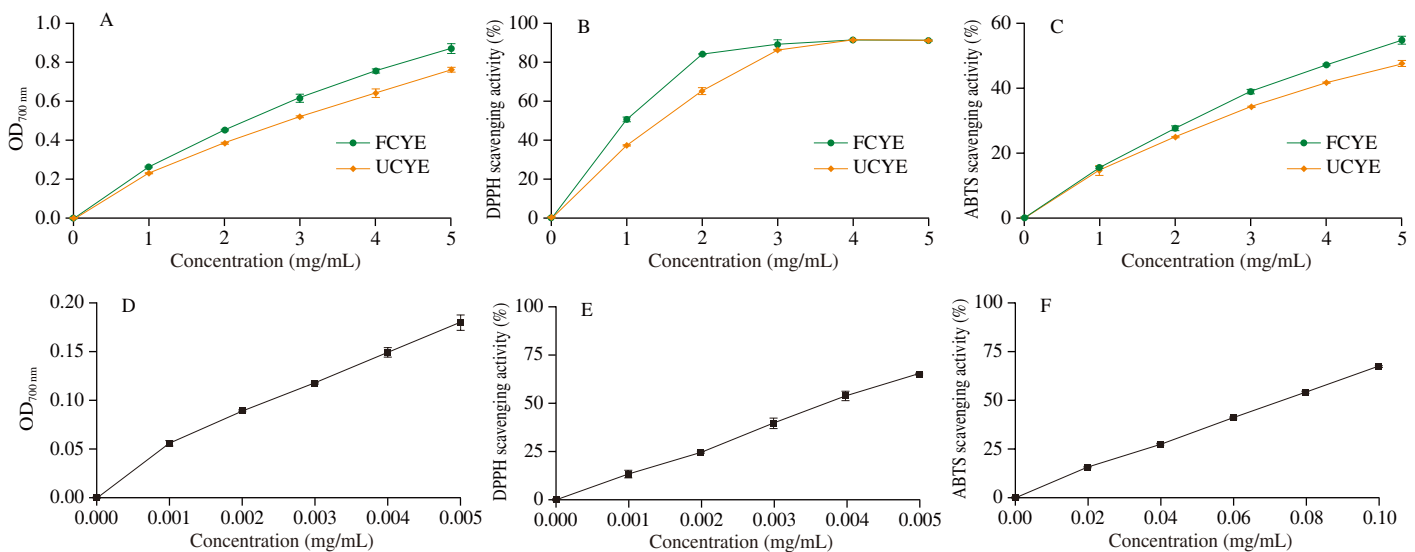


Fig. 3 Antioxidant activities *in vitro*. (A) TRP of FCYE and UCYE; (B) DPPH radical scavenging activities of FCYE and UCYE; (C) ABTS radical scavenging activities of FCYE and UCYE; (D) TRP of VC; (E) DPPH radical scavenging activity of VC; (F) ABTS radical scavenging activity of VC. Data are represented as means $\pm$ SD ($n = 3$).

Various studies have shown that microbial fermentation could improve the bioactive components and antioxidant activities of foods^[36–42]. For example, the black carrot juices fermented by *S. boulardii* were rich in polyphenols and antioxidant activity^[42]. The biotransformation of blueberries during the fermentation process improved its TPC and bioavailability, and then increased its antioxidant activity^[37]. The contents of ascorbic, galli, and *p*-coumaric acids in pearl millet increased remarkably with fermentation, thus enhancing the antioxidant activity of pearl millet extracts^[38]. Meanwhile, the antioxidant properties of red bean fermented by *Bacillus subtilis* could also be significantly enhanced^[39]. Likewise, the polyphenol extract from fermented soybean with *S. cerevisiae* exhibited stronger TRP and DPPH free radical scavenging activity^[40]. Moreover, *S. boulardii* fermentation of wort-based beverages containing grapefruit (*Citrus paradisi* L.) or tangerine (*Citrus reticulata* L.) zest essential oil also possessed superior antioxidant activity^[41]. In brief, the excellent antioxidant activities of FCYE makes it promising in the development and research of natural antioxidants.

3.6 Immunomodulatory activities of FCYE

As one of the important components of the innate immune system, macrophages play a key role in the inflammatory response and host defense^[43]. From Fig. 4, it was detected about the immunomodulatory activities of FCYE on RAW264.7 cells, from cell viability, phagocytosis activity and NO production ability, respectively.

As shown in Fig. 4A, in the concentration range of 1.00–50.00 µg/mL, FCYE obviously promoted the proliferation activity of RAW264.7 cells, while less effects were displayed by UCYE. In terms of phagocytosis activity, it could be measured by neutral red that entered cells by endocytosis because of its characteristic of macromolecule and lipophilic dye. Results showed that FCYE and UCYE exhibited similar improving abilities (Fig. 4B). Additionally, NO produced in activated macrophages mainly aimed at inhibiting the invasion of foreign pathogens, thereby playing an immunomodulatory role. From Fig. 4C, compared with the blank control (2.85 ± 0.10 µmol/L), the NO production in cells treated by FCYE at 50.00 µg/mL increased significantly to (5.18 ± 0.36 µmol/L ($P < 0.001$), while that of UCYE was almost unchanged at the same concentration ((3.03 ± 0.41) µmol/L, $P > 0.05$). Comprehensive analysis showed that FCYE owned superior immunomodulatory activity than UCYE, especially for cell proliferation activity and NO production ability. Research found that many compounds from Chinese yam (including dioscorin, polysaccharides and glycoprotein) had favorable immunomodulatory activity^[44]. Also, the immunomodulatory properties of plant-based fermented foods were well documented^[45–47]. For example, fermented blueberries and blackberries could protect against chronic disorders by improving immunomodulatory activity^[46]. *Monascus*-fermented *Dioscorea* products also indicated decent antioxidant and immunomodulatory potential^[47].

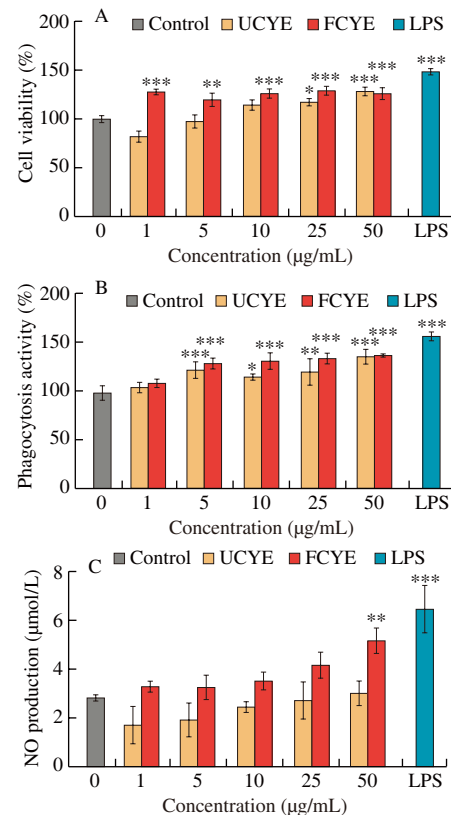


Fig. 4 Immunomodulatory activities of FCYE on the cell viability (A), phagocytosis activity (B), and NO production (C) of RAW264.7 macrophage cells. Lipopolysaccharide (LPS) was used as a positive control. Data are represented as means $\pm$ SD ($n = 5$). The statistically significant differences with the control group were represented as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

4. Conclusion

In this study, Chinese yam fermentation by *S. boulardii* was studied based on UPLC-MS/MS and related differential metabolites and biosynthetic pathways were also analyzed, along with further bioactivities exploration of FCYE. Results showed that organic acids and flavonoids could discriminate the fermented sample from the control and they were considered to exert an important effect in the improvement of functional bioactivities. Furthermore, compared with UCYE, FCYE indeed presented better *in vitro* antioxidant activity and immunomodulatory activity. Through understanding the metabolic composition and potential bioactivities of fermented Chinese yam, this study could provide a theoretical basis for ulterior researches on the health benefits of probiotic-fermented products, which might also be extended to other fermented food.

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.

Acknowledgments

This study was supported by National Natural Science Foundation of China (32172211), the Natural Science Foundation

of Henan Province for Outstanding Youth, China (202300410365), the National Key Research and Development Program of China (2022YFF1103300), the Program for Science and Technology Innovation Talents in Universities of Henan Province (22HASTIT037) and the Technology Development (Cooperation) project of Zhengzhou University (20210442A, 20210327A).

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