# Effect of Solid-State Fermentation on Nutritional Quality of Leaf Flour of The Drumstick Tree (Moringa Oleifera Lam.)

**Honghui Shi**
South China Agricultural University
**Endian Yang**
South China Agricultural University
**Yun Li**
Beijing Forestry University
**Xiaoyang Chen**
South China Agricultural University
**Junjie Zhang** ( zhangjunjie3168@163.com )
South China Agricultural University https://orcid.org/0000-0002-4780-3906

**Research**

**Keywords:** Moringa oleifera Lam., solid-state fermentation, nutritional quality, antioxidant activity

**Posted Date:** November 9th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-102471/v1

# Abstract

**Background:** The drumstick tree is a fast-growing multipurpose tree with a large biomass and high nutritional value. However, it has rarely been exploited as a protein source. This study investigated solid-state fermentation induced by *Aspergillus niger*, *Candida albicans* and *Bacillus subtilis* to obtain high-quality protein feed from drumstick leaf flour.

**Results:** Solid-state fermentation induced significant changes in the nutritional composition of drumstick leaf flour. The concentrations of crude protein, small peptides and amino acids increased significantly after fermentation. The protein profile was also affected by the fermentation process. Macromolecular proteins in drumstick leaf flour were degraded, whereas other high molecular weight proteins were increased. However, the concentrations of crude fat, fiber, total sugar and reducing sugar were decreased, as were the anti-nutritional factors tannins, phytic acid and glucosinolates. After 24 h fermentation, the concentrations of total phenolics and flavonoids were increased. The antioxidant capacity was also significantly enhanced.

**Conclusions:** The results suggested that the feed quality of drumstick leaves was greatly improved by solid-state fermentation.


# Introduction

The demand for animal protein for human nutrition is still rising in the developing world and the cost of feed for livestock is also increasing [1]. Therefore, a new source of feed protein is urgently needed to solve these problems. Among the available forage crops, special focus has been given to the effects of *Moringa oleifera* Lam. on livestock growth and production [2–4]. *Moringa oleifera* Lam., commonly known as the drumstick tree, belongs to the family of Moringaceae and is widely distributed in tropical and subtropical regions [5, 6]. It is extremely nutritious, containing high levels of protein, vitamins, minerals and phytochemicals [5]. These nutritional traits together with its high production of leaf mass and adaptability to climate conditions and dry soils make drumstick a potential high quality feed source for livestock [7]. Dietary inclusion of drumstick has been shown to enhance nutritional status, growth performance, milk production and meat quality in several livestock species [2, 4, 8]. Such characteristics show that drumstick is a rich source of nutrients and biological activities for livestock, which could help to relieve the shortage of feed resources and decrease the need for antibiotics. However, anti-nutritional factors (ANF) in drumstick, such as tannins, phytic acid and glucosinolates, could affect the palatability, digestion and absorption, limiting its nutrition availability [9]. Moreover, most of the proteins are insoluble despite drumstick tree leaves having a relatively high protein content [10]. So far, the nutritional value of drumstick leaves has mainly been improved by heating, grinding, cooking and other physical and chemical means [11–13]. Although some of the ANF can be removed, the nutrient content may also be destroyed by these processes. Therefore, a more suitable process for improving drumstick feed quality is needed.

Solid-state fermentation (SSF) involves the growth of microorganisms on substrates with limited water content [14]. Numerous studies have demonstrated that the functionalities of various agricultural by-products can be enhanced by SSF. Indeed, many beneficial compounds have been produced through SSF, such as organic acids, enzymes, aromatic and flavor compounds, as well as bioactive compounds [14–16]. In recent years, SSF has been widely used in the feedstock industry and has shown good prospects for promoting nutrient utilization and decreasing ANF levels [17, 18]. Although, several studies have been conducted on the SSF of drumstick leaf flour (DLF), they mainly focused on the selection of strains, optimization of fermentation technology and evaluation of the nutritional value before and after fermentation [18, 19]. Only a few studies have examined the dynamic changes of nutrients and antioxidant components in the fermentation process, and even fewer have reported on the antioxidant activity of DLF after fermentation.

This study evaluated SSF processes induced by *Aspergillus niger*, *Candida albicans* and *Bacillus subtilis* for the mixed fermentation of drumstick leaves. Effects on the nutrient composition, physical and chemical properties and functional activity of drumstick leaves were studied. The main aims were to obtain a high nutrition, multi-functional feed, broaden the utilization ways of drumstick feed and provide some theoretical basis for the further processing of drumstick resources.

# Materials And Methods

### Microorganisms

*Aspergillus niger* strain GIM 3.576 and *Candida utilis* strain GIM 1.427 were obtained from the Guangdong Microbial Strain Preservation Center. *Bacillus subtilis* CICC 31188 was obtained from the China Center of Industrial Culture Collection (CICC). GIM 3.576 was cultured on potato dextrose agar (PDA) slants for 3 days at 28 ℃, GIM 1.427 was cultured on yeast extract peptone dextrose (YPD) slants for 3 days at 28 ℃ and CICC 31188 was cultured on Luria-Bertani (LB) slants for 2 days at 30 ℃. After being activated for 3 generations, GIM 3.576 was cultured in PDA solid medium until mycelia had spread over the entire petri dish. The spores were then rinsed with sterile saline solution, and the concentration of the spore suspension was calculated and diluted to $1\times10^7$ cfu/mL on a blood cell counting board. GIM 1.427 was incubated in YPD liquid medium at 28 ℃ and 150 r/min for 3 days, then diluted to a concentration of $1\times10^8$ cfu/mL. CICC 31188 was cultured in LB liquid medium at 30 ℃ and 250 r/min and diluted to a concentration of about $1\times10^8$ cfu/mL.

### Solid-state fermentation

Drumstick leaf samples were harvested from trees of the PKM-1 cultivar. After harvesting, the samples were sun-dried until a constant weight was reached, then ground to a powder and passed through a 40-mesh sieve. Portions of 50 g were packed and sealed in polypropylene bags for sterilization in an autoclave at 121 ℃ for 20 min. After cooling, sterilized water was added to adjust the initial water content to 50%. Then 6% (v/w) of the GIM 3.576 spore suspension was inoculated onto each DLF. After

fermentation for 24 h, 6% (v/w) of the GIM 1.427 suspension and 12% (v/w) of the CICC 31188 suspension were inoculated simultaneously and fermented in a biochemical incubator at 32 ℃ for a total of 7 days. Some of the fermentation samples were stored at -20 ℃ for detection of the active substances, whereas the rest were dried at 50 ℃, crushed and screened for chemical analysis.

### Scanning electron microscopy

Samples were prepared by a standard procedure of alcohol dehydration for scanning electron microscopy (SEM, Carl Zeiss, EVO MA15, Germany) analysis. After dehydration, the samples were placed on an aluminum column and sprayed with 12 nm of gold using ion sputtering coater (Leica, EM ACE 600, Germany). SEM images were recorded at 25 kV and with high vacuum modes.

### Protein and amino acid analysis

The crude protein (CP) concentration was analyzed using methods of the Association of Official Analytical Chemists [20]. The protein profile was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Zuo et al. with slight modifications [21]. Proteins from unfermented and fermented samples were extracted using the plant total protein extraction kit (KeyGen Biotech, China), denatured with 2×Tris-glycine SDS loading buffer and separated using a 12% gel for SDS-PAGE at 110 V. The gel was stained with Coomassie Brilliant Blue R250 and de-stained with 8% acetic acid until the protein bands were visible. The amino acid (AA) profile was analyzed using an automatic AA analyzer (L-8900, Hitachi, Tokyo, Japan). Trichloroacetic acid-soluble protein (TCA-SP) was analyzed as reported by Ovissipour et al. [22]. Small peptide concentration was calculated by subtracting the concentration of free amino acids from that of TCA-SP.

### Chemical analysis

Dry matter (DM), ether extract (EE), crude fiber (CF), crude ash (CA), calcium (Ca) and phosphorus (P) were analyzed using methods of the Association of Official Analytical Chemists [20]. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were determined by methods described by Van Soest et al. [23]. Potassium (K), sodium (Na), magnesium (Mg), copper (Cu), zinc (Zn) and iron (Fe) were determined by atomic absorption spectrophotometry as described by the AOAC [20]. Total sugars and reducing sugars were determined by 3,5-dinitrosalicylic acid colorimetry [24]. Phytic acid was determined according to the method described by Gao et al. [25]. Glucosinolates were determined by palladium chloride colorimetry as described by Hu et al. [26].

### Determination of total phenolic, tannin and flavonoid content

A methanol extract from each sample powder was prepared according to the method described by Zuo et al. [27]. The lyophilized samples were extracted twice with 80% (v/v) methanol for 3 h at room temperature and filtered through a 0.45 μm injection filter. The total phenolic concentration of the methanol-extracted samples was determined using the Folin-Ciocalteu procedure and expressed as grams of gallic acid equivalents (GAE) in 100 g of the extract [28]. The concentration of tannins was

calculated as the difference between the concentrations of total phenols and simple phenols after removing tannin from the extract using insoluble polyvinylpyrrolidone. The total flavonoid concentration was analyzed using the aluminum chloride colorimetric method and expressed as grams of rutin equivalents (RUE) in 100 g of the extract [29].

### Determination of antioxidant capacity

The $ABTS^{+}$ (2,2'-azino-bis (3-ethylbenzothiazoline6-sulfonic acid) radical cation) radical-scavenging capacity of methanol extracts of samples were evaluated according to the method of Sasipriya and Siddhuraju with slight modifications [30]. The stock solution was prepared by reacting 5 mL of 7 mM ABTS with 88 µL of 40 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The stock solution was diluted with methanol to give an absorbance of about 0.700 ± 0.020 at 734 nm. To determine the antioxidant capacity, 0.5 mL of methanolic extract samples (final concentration 1 mg/mL) and 4.0 mL of stock solution were mixed and incubated for 6 min. The absorbance was monitored at 734 nm.

The DPPH (2,2-diphenil-1-picrylhydrazyl) radical-scavenging capacity of methanol extracts of samples was evaluated according to the method of Arora and Chandra (2010) with slight modifications [31]. A 100 mM solution of DPPH was prepared in absolute methanol, and 2.0 mL of the DPPH solution was added to 0.5 mL of a methanolic extract of a sample (final concentration 1 mg/mL). After thorough mixing, the solution was kept in the dark at room temperature for 30 min. The absorbance of the samples was measured using an UV-visible spectrophotometer at 517 nm against a methanol blank. Each sample was tested three times and the values were averaged.

The free radical scavenging activity (RSA) was calculated as a percentage using the following equation:
$RSA\ (\%) = [1-(Ai-Aj)/Ae] \times 100\%$

Where Ae is the absorbance of the control, Ai is the absorbance of the sample extract and Aj is the absorbance of the control of sample.

The total antioxidant capacity was determined using the ferric reducing ability of plasma antioxidant power (FRAP) assay. The procedure was conducted according to the manufacturer's instruction for the T-AOC kit (Jiancheng Bioengineering Institute, Nanjing, China).

### Statistical analysis

All processing treatments were performed in triplicate. The statistical analysis was carried out using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA), Duncan's multiple range test was used to detect differences among means. A $p$-value < 0.05 was considered significant. Pearson's correlation analysis was also performed.

## Results And Discussion

## Microscopic observation

During the fermentation process, the color of DLF changed from green to dark brown（Figure 1） accompanied by a sweet smell. The microstructure was clearly different between fermented DLF (FDLF) and unfermented DLF (UDLF) (Figure 1). An irregularly shaped and rough surface was mainly observed in FDLF, whereas the microstructure of DLF was more regular and the surface smooth. This change of microstructure may increase the surface area of the substrate and facilitate the full reaction between enzyme and substrate.

## Effect of solid-state fermentation on proteins and amino acids

The protein content is the most important parameter that determines the overall quality of animal feed products. It is well known that the protein content can vary depending on the microorganism used and their carbon and nutrient accessibility, as well as the cultivation conditions, such as carbon and nutrient sources, water content and pH. The analysis showed that the average concentration of CP in the raw flour was 28.42% (Table 1), close to the figure reported by Teixeira et al. [10] but lower than the values reported by Moyo and Masika [32], most likely due to differences in the growth stage and planting conditions [33]. SSF significantly increased the CP concentrations from 28.42% to 40.98% (Table 1). The enrichment of CP could be the result of increased fungal biomass, suggesting that the treated substrate could act as a good protein source for livestock. However, it could also be due to the concentration effect caused by the aggravation of dry matter loss.

To analyze the influence of fermentation on the DLF protein profile, SDS-PAGE was performed. SSF affected the characteristics of proteins in DLF. The molecular weight of the main protein fractions in the unfermented DLF was 55 kDa (Figure 2). The maximal degradation of large proteins in the FDLF was almost complete after 24 h of fermentation. This was likely because highly active proteases were able to decompose the large proteins secreted by the microorganisms during fermentation [17]. This reduction of protein sizes is important to increase the digestibility of protein. However, from the third day of fermentation, the color of the protein bands gradually deepened and bands appeared at around 33-45 kDa and 60-140 kDa, similar to the results reported by Zuo et al. [21]. This could be due to the production of single-celled proteins in the late fermentation period. This phenomenon indicated that fermentation not only degraded the macromolecular proteins in DLF but also increased the abundance of other proteins with high molecular weight.

Drumstick leaves contain all essential amino acids, but the content varies greatly depending on growth environments, cultivation mode, tree age, leaf maturity and other conditions [34, 35]. Evaluation of the amino acid profile confirmed significant differences between the fermented substrate and raw flour (Table 2). The levels of most amino acids increased, whereas glutamic acid and lysine decreased. The alterations in amino acid profiles may vary depending on the microorganism used [36]. The inoculated microorganisms may use glutamic acid and lysine for metabolic activity, resulting in a reduction in their concentrations in the fermented substrate compared to those in raw flour. After fermentation, the total amino acid content of DLF was 24.88%, which was significantly higher than that of the unfermented

substrate. This may be due to enzymatic hydrolysis of large proteins into small amino acids or the microbial synthesis of some amino acids. The concentration of essential amino acids and non-essential amino acids in the fermented substrate also increased, by 1.81% and 1.76%, respectively. Animals not only have dietary requirements for essential amino acids but also need nutritionally nonessential amino acids to achieve maximum growth and production performance. Therefore, increasing non-essential amino acids in animal feeds is beneficial. It has been reported that peptides are more rapidly utilized than proteins and amino acids [37]. The small peptide concentration in our study increased from 5.72% to 12.03% after fermentation. The increased peptide content may positively affect the bioactivity of DLF because they may contribute to antioxidative and metal-chelating activities [17].

### Effect of solid-state fermentation on the chemical composition

The results regarding the chemical composition of the fermented substrate and raw flour are shown in Table 1. SSF increased the content of crude ash, neutral washing fiber and lignin. In addition, the CF concentration of the fermented substrate was decreased markedly, by 70.07%, compared to the control. There were also clear reductions in total sugars and reducing sugars from 18.49% and 12.38% to 5.34% and 1.69%, respectively (Table 1). Meanwhile, the ether extract (fat) concentration decreased by 30.94% compared with that before fermentation. This suggests that the microbial fermentation process consumed carbohydrates and fats, especially small molecules of sugars. At the same time, SSF resulted in an increase in the mineral content, probably due to the metabolic activity of the microorganisms or dry matter loss.

### Effect of solid-state fermentation on total flavonoid, phenolic content and antioxidant capacity

Antioxidants are free radical scavengers that can protect the body from free radicals that can cause a variety of diseases, including ischemia, asthma, anemia, dementia and arthritis. Phenols and flavonoids are considered to be some of the safest natural antioxidants, and fermentation technology is an effective way to increase the concentration of these compounds. In this study, the concentration of total phenols and flavones reached the maximum value on the first day of fermentation. Afterwards, the content of total phenols and flavonoids decreased as the fermentation continued (Figure 3). Dey et al. [38] proposed that the reason for the increased content was that various extracellular enzymes secreted by microorganisms destroy the intact cell wall structure of plants, releasing flavonoids from within cells and phenols bound to the cell walls during fermentation. At the same time, microorganisms can produce some phenols through secondary metabolism. However, prolonged fermentation may lead to the diffusion and oxidation of phenolic substances.

It is crucial to evaluate the antioxidant potential of extracts using more than one method due to the different mechanisms of antioxidant activity. In the present study, the antioxidant activity of fermented substrates was measured using $ABTS^{+}$, DPPH free radical scavenging methods and FRAP. The antioxidant activity showed a trend of first increasing and then decreasing with increasing fermentation time (Figure 3), similar to results obtained with okra seeds during fermentation by Adetuyi and Ibrahim

[39]. The antioxidant activity of DLF after fermentation was slightly lower than that before fermentation. Hossain et al. [40] suggested that this might be because of a too long fermentation time and reduced content of phenols and other substances. Many studies have shown that the antioxidant capacity of plants is directly related to the content of phenolic compounds and flavones. In this study, Pearson's correlation analysis showed that flavonoids were only slightly positively correlated with DPPH radical scavenging rate and total antioxidant capacity, whereas total phenols were highly positively correlated with the three antioxidant indexes tested (Table 3), indicating that the total phenols and flavonoid concentrations of FDLF were closely related to antioxidant activity. However, total phenols and flavonoids are not the only factors affecting antioxidant activity. Small peptides and amino acids, such as leucine, methionine, tyrosine, histidine, and tryptophan, can also make the fermented sample more reductive. In addition, determination of phenolic substances with the Folin-Ciocalteu reagent may be influenced by a variety of non-phenolic compounds, such as reducing sugars, aromatic amino acids and citric acids, which often do not have free radical scavenging capacity. In general, the synergistic effect between phenols and other components in the solid fermentation of DLF may be the main reason for this phenomenon.

### Effect of solid-state fermentation on anti-nutritional factors

Tannins, phytic acid and glucosinolates are the main anti-nutrients in DLF [41-43]. Tannins can precipitate proteins, amino acids, alkaloids and other organic molecules in aqueous solution, which hinder the absorption of some nutrients due to complexation. Further, the bitter taste of tannins may affect the palatability of feed. High concentrations of tannins have an adverse effect on animal productivity and digestibility. Phytic acid is an organic acid that cannot be digested by animals with a single stomach. Therefore, phytic acid is eventually expelled in an animal's feces, which may be degraded by aquatic microorganisms after entering water, releasing phosphorus and resulting in serious eutrophication of water [44]. Although glucosinolates are non-toxic, they may decompose into glucose, isothiocyanate, nitrile, thiocarcinate and other toxic compounds through endogenous myrosinase. Their main anti-nutritional effects include reducing the palatability of feed, inducing iodine deficiency and damaging liver and kidney function. They are also more harmful to non-ruminants than ruminants [45]. Nevertheless, phytase, tannase and other enzymes can degrade these substances. *Aspergillus* is considered to be the main source of these enzymes. In this study, the phytic acid and tannin concentrations of DFL were 18.31 mg/g and 14.60 mg/g, respectively, which are close to the values of 22.3 mg/g and 16.3 mg/g reported by Stevens et al. [9]. After fermentation, the levels of tannins, phytic acid and glucosinolates were drastically decreased (Table 1). This might have been caused by the secretion of tannase, phytase and other biological enzymes, which are able to break down tannins, phytic acid and glucosinolates.

## Conclusion

In this study, SSF by *Aspergillus niger*, *Candida albicans* and *Bacillus subtilis* was shown to be beneficial for improving the nutritional value of DLF. It not only increased the content of nutrients, such as CPs and

small peptides, but also decreased the content of anti-nutrient factors, such as phytic acid and tannins. Our results suggest that the SSF method offers an effective approach for improving the quality of unconventional proteins sources such as DLF.

# Declarations

### Ethics approval and consent to participate

Compliance with ethical standards.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

No potential conflict of interest is reported by the authors.


### Funding

This work was funded by the Forestry Technology Innovation Program, Department of Forestry of Guangdong Province (2018KJCX001); the Characteristic Innovation Program (Natural Science), the Department of Education of Guangdong Province (2018KTSCX018); and the Youth Innovation Program (Natural Science), Department of Education of Guangdong Province (2019KQNCX010).


### Authors' contribution

CX and ZJ conceived and designed research; SH conducted experiments; SH and YE collected and analyzed the data; ZJ and CX wrote the manuscript; ZJ and CX obtained fundings.


### Acknowledgements

Not applicable.


# References


1. Tufarelli V, Ragni M, Laudadio V. Feeding forage in poultry: a promising alternative for the future of production systems. Agriculture. 2018;8(6): 81.
2. Cui YM, Wang J, Lu W, Zhang HJ, Wu SG. Effect of dietary supplementation with *Moringa oleifera* leaf on performance, meat quality, and oxidative stability of meat in broilers. J. Poult. Sci.

2018;97(8):2836–44.

3. Yusuf AO, Mlambo V, Iposu SO. A nutritional and economic evaluation of *Moringa oleifera* leaf meal as a dietary supplement in West African dwarf goats. S. Afr. J. Anim. Sci. 2018;48(1): 81–7.
4. Zeng B, Sun JJ, Chen T, Sun BL, He Q, Chen XY, et al. Effects of *Moringa oleifera* silage on milk yield, nutrient digestibility and serum biochemical indexes of lactating dairy cows. J. Anim. Physiol. Anim. Nutr. 2018;102(1):75–81.
5. Leone A, Spada A, Battezzati A, Schiraldi A, Aristil J, Bertoli S. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: an overview. Int. J. Mol. Sci. 2015;16:12791–835.
6. Popoola JO, Obembe OO. Local knowledge, use pattern and geographical distribution of *Moringa oleifera* Lam. (Moringaceae) in Nigeria. J. Ethnopharmacol. 2013;150:682–91.
7. He L, Lv H, Chen N, Wang C, Zhang Q. Improving fermentation, protein preservation and antioxidant activity of *Moringa oleifera* leaves silage with gallic acid and tannin acid. Bioresour. Technol. 2020;297:122390.
8. Kholif AE, Gouda GA, Olafadehan OA, Abdo MM. Effects of replacement of *Moringa oleifera* for berseem clover in the diets of Nubian goats on feed utilisation, and milk yield, composition and fatty acid profile. Animal. 2018;12(5):964–72.
9. Stevens CG, Ugese FD, Otitoju GT, Baiyeri KP. Proximate and anti-nutritional composition of leaves and seeds of *Moringa oleifera* in Nigeria: a comparative study. Agro-Science. 2016;14(2): 9–17.
10. Teixeira EMB, Carvalho MRB, Neves VA, Silva MA, Arantes-Pereira L. Chemical characteristics and fractionation of proteins from *Moringa oleifera* Lam. leaves. Food Chem. 2014;147: 51–54.
11. Adedapo AA, Falayi OO, Oyagbemi AA. Evaluation of the analgesic, antiinflammatory, antioxidant, phytochemical and toxicological properties of the methanolic leaf extract of commercially processed *Moringa oleifera* in some laboratory animals. J. Basic Clin. Physiol. Pharmacol. 2015; 26 (5):491–9.
12. Phatsimo GM, Cukrowska E, Chimuka L. Development of pressurized hot water extraction (PHWE) for essential compounds from *Moringa oleifera* extracts. Food Chem. 2015;172:423–7.
13. Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. Maximizing total phenolics, total flavaonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. Ind. Crops Prod. 2013;44:566–71.
14. Dulf FV, Vodnar DC, Dulf EH, Pintea A. Phenolic compounds, flavonoids, lipids and antioxidant potential of apricot (*Prunus armeniaca* L.) pomace fermented by two filamentous fungal strains in solid state system. Chem. Cent. J. 2017;11(1): 92.
15. Bennett P, Yang ST. Beneficial effect of protracted sterilization of lentils on phytase production by *Aspergillus ficuum* in solid state fermentation. Biotechnol. Prog. 2012;28(5):1263–70.
16. Jin B, Zepf F, Bai Z, Gao B, Zhu N. A biotech-systematic approach to select fungi for bioconversion of winery biomass wastes to nutrient-rich feed. Process Saf. Environ. Prot. 2016;103: 60–8.

17. Chi CH, Cho SJ. Improvement of bioactivity of soybean meal by solid-state fermentation with *Bacillus amyloliquefaciens* versus Lactobacillus spp. and Saccharomyces cerevisiae. LWT - Food Sci. Technol. 2016;68:619–25.
18. Wang J, Cao F, Su E, Zhao L, Qin W. Improvement of animal feed additives of ginkgo leaves through solid-state fermentation using *Aspergillus niger*. Int. J. Biol. Sci. 2018;14(7):736.
19. Zhang M, Huang Y, Zhao H, Wang T, Xie C, Zhang D. Solid-state fermentation of *Moringa oleifera* leaf meal using Bacillus pumilus CICC 10440. J. Chem. Technol. Biotechnol. 2017;92(8):2083–89.
20. AOAC. Official methods of analysis of the AOAC international. Gaithersburg: Association of Official Analytical Chemists International. 2005.
21. Zuo SS, Niu DZ, Ning TT, Zheng ML, Jiang D. Protein enrichment of sweet potato beverage residues mixed with peanut shells by *Aspergillus oryzae* and *Bacillus subtilis* using central composite design. Waste Biomass Valori. 2017; 9(5):835–44.
22. Ovissipour M, Abedian A, Motamedzadegan A, Rasco B, Safari R, Shahiri H. The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. Food Chem. 2009;115 (1):238–42.
23. Van Soest PV, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 1991; 74(10):3583–97.
24. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 1959;31:426–8.
25. Gao Y, Shang C, Maroof MAS, Biyashev RM, Buss GR. A modified colorimetric method for phytic acid analysis in soybean. Crop Sci. 2007;47(5):1797–803.
26. Hu Y, Liang H, Yuan Q, Hong Y. Determination of glucosinolates in 19 Chinese medicinal plants with spectrophotometry and high-pressure liquid chromatography. Nat. Prod. Res. 2010;24(13):1195–205.
27. Zuo Y, Chen H. Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta. 2002;57(2):307–16.
28. Generalić Mekinić I, Skroza D, Ljubenkov I, Šimat V, Smole Možina S, Katalinić V. In vitro antioxidant and antibacterial activity of Lamiaceae phenolic extracts: a correlation study. Food Technol. Biotechnol. 2014;52(1):119–27.
29. Lin J, Tang C. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 2007;101(1):140–7.
30. Sasipriya G, Siddhuraju P. Effect of different processing methods on antioxidant activity of underutilized legumes, Entada scandens seed kernel and Canavalia gladiata seeds. Food Chem. Toxicol. 2012;50(8):2864–72.
31. Arora DS, Chandra P. Assay of antioxidant potential of two aspergillus isolates by different methods under various physio-chemical conditions. Braz. J. Microbiol. 2010; 41(3):765–77.

32. Moyo B, Masika P. Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. Afr. J. Biotechnol. 2011;10(60):12925–33.
33. Mendieta-Araica B, Spörndly E, Reyes-Sánchez N, Salmerón-Miranda F, Halling M. Biomass production and chemical composition of *Moringa oleifera* under different planting densities and levels of nitrogen fertilization. Agroforest. Syst. 2013;87(1): 81–92.
34. Ndubuaku UM, Nwankwo VU, Baiyeri KP. Influence of poultry manure application on the leaf amino acid profile, growth and yield of moringa (*Moringa oleifera* Lam.) plants. Albanian J. Agric. Sci. 2014;13(1):42.
35. Olaofe O, Adeyeye EI, Ojugbo S. Comparative study of proximate, amino acids and fatty acids of *Moringa oleifera* tree. Elixir Appl. Chem. 2013;54:12543–54.
36. Medeiros S, Xie J, Dyce PW, Cai HY, Delange K, Zhang H, et al. Isolation of bacteria from fermented food and grass carp intestine and their efficiencies in improving nutrient value of soybean meal in solid state fermentation. J. Anim. Sci. Biotechno. 2018;9: 29.
37. Kodera T, Hara H, Nishimori Y, Nio N. Amino acid absorption in portal blood after duodenal infusions of a soy protein hydrolysate prepared by a novel soybean protease D3. J. Food Sci. 2006;71(7):S517–25.
38. Dey TB, Chakraborty S, Jain KK, Sharma A. Antioxidant phenolics and their microbial production by submerged and solid state fermentation process: a review. Trends Food Sci. Technol. 2016;53: 60–74.
39. Adetuyi FO, Ibrahim TA. Effect of fermentation time on the phenolic, flavonoid and vitamin C contents and antioxidant activities of okra (*Abelmoschus esculentus*) seeds. Nigerian Food J. 2014;32(2):128–37.
40. Hossain A, Khatun A, Munshi MK, Hussain MS, Huque R. Study on antibacterial and antioxidant activities of raw and fermented *Moringa oleifera* Lam. leaves. J. Microbiol. Biotechnol. Res. 2017;6(4):23–9.
41. Amaglo NK, Bennett RN, Lo Curto RB, Rosa ES, Turco VL. Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. Food Chem. 2010;122(4):1047–54.
42. Chodur GM, Olson ME, Wade KL, Stephenson KK, Nouman W, Garima, et al. Wild and domesticated *Moringa oleifera* differ in taste, glucosinolate composition, and antioxidant potential, but not myrosinase activity or protein content. Sci. Rep. 2018;8(1): 7995.
43. Ogbe AO, Affiku JP. Proximate study, mineral and anti-nutrient composition of *Moringa oleifera* leaves harvested from Lafia, Nigeria: potential benefits in poultry nutrition and health. J. Microbiol. Biotechnol. Food Sci. 2011;1(3):296.
44. Singh B, Satyanarayana T. Microbial phytases in phosphorus acquisition and plant growth promotion. Physiol. Mol. Biol. Plants. 2011;17(2):93–103.
45. Tripathi MK, Mishra AS. Glucosinolates in animal nutrition: a review. Anim. Feed Sci. Technol. 2007;132(1-2):1–27.

# Tables

Table 1 Nutrient composition of FDLF and UDLF.

| Item | UDLF | FDLF |
|---|---|---|
| CP, % | 28.42±1.17$^{b}$ | 40.98±0.59$^{a}$ |
| Small peptides, % | 5.72±0.20$^{b}$ | 12.03±0.12$^{a}$ |
| EE, % | 5.43±0.18$^{a}$ | 3.75±0.15$^{b}$ |
| CF, % | 7.25±0.02$^{a}$ | 2.17±0.04$^{b}$ |
| Ash, % | 10.49±0.03$^{b}$ | 16.75±0.02$^{a}$ |
| Total sugars, % | 18.49±0.62$^{a}$ | 5.34±0.88$^{b}$ |
| Reducing sugars, % | 12.38±0.81$^{a}$ | 1.69±0.16$^{b}$ |
| NDF, % | 24.41±2.11$^{b}$ | 38.61±2.97$^{a}$ |
| ADF, % | 13.40±2.06 | 12.97±2.75 |
| Lignin, % | 1.30±0.16$^{b}$ | 2.13±0.38$^{a}$ |
| Hemicellulose, % | 11.01±0.29$^{b}$ | 22.40±1.68$^{a}$ |
| Cellulose, % | 12.62±1.49$^{a}$ | 7.50±0.84$^{b}$ |
| Ca, % | 2.34±0.01$^{b}$ | 3.64±0.06$^{a}$ |
| P, % | 0.25±0.00$^{b}$ | 0.42±0.01$^{a}$ |
| K, % | 1.04±0.02$^{b}$ | 1.28±0.01$^{a}$ |
| Na, % | 0.03±0.01$^{b}$ | 0.12±0.01$^{a}$ |
| Mg, % | 0.96±0.02$^{b}$ | 1.31±0.02$^{a}$ |
| Fe, mg/100 g | 41.70±0.57$^{b}$ | 73.05±1.06$^{a}$ |
| Cu, mg/100 g | 1.25±0.01$^{b}$ | 1.81±0.01$^{a}$ |
| Zn, mg/100 g | 2.45±0.09$^{b}$ | 3.53±0.06$^{a}$ |
| Tannins, mg/g | 14.60±0.58$^{a}$ | 8.59±0.45$^{b}$ |
| Phytic acid, mg/g | 18.31±0.71$^{a}$ | 7.30±0.74$^{b}$ |
| Glucosinolates, mg/g | 19.11±0.13$^{a}$ | 14.72±0.24$^{b}$ |

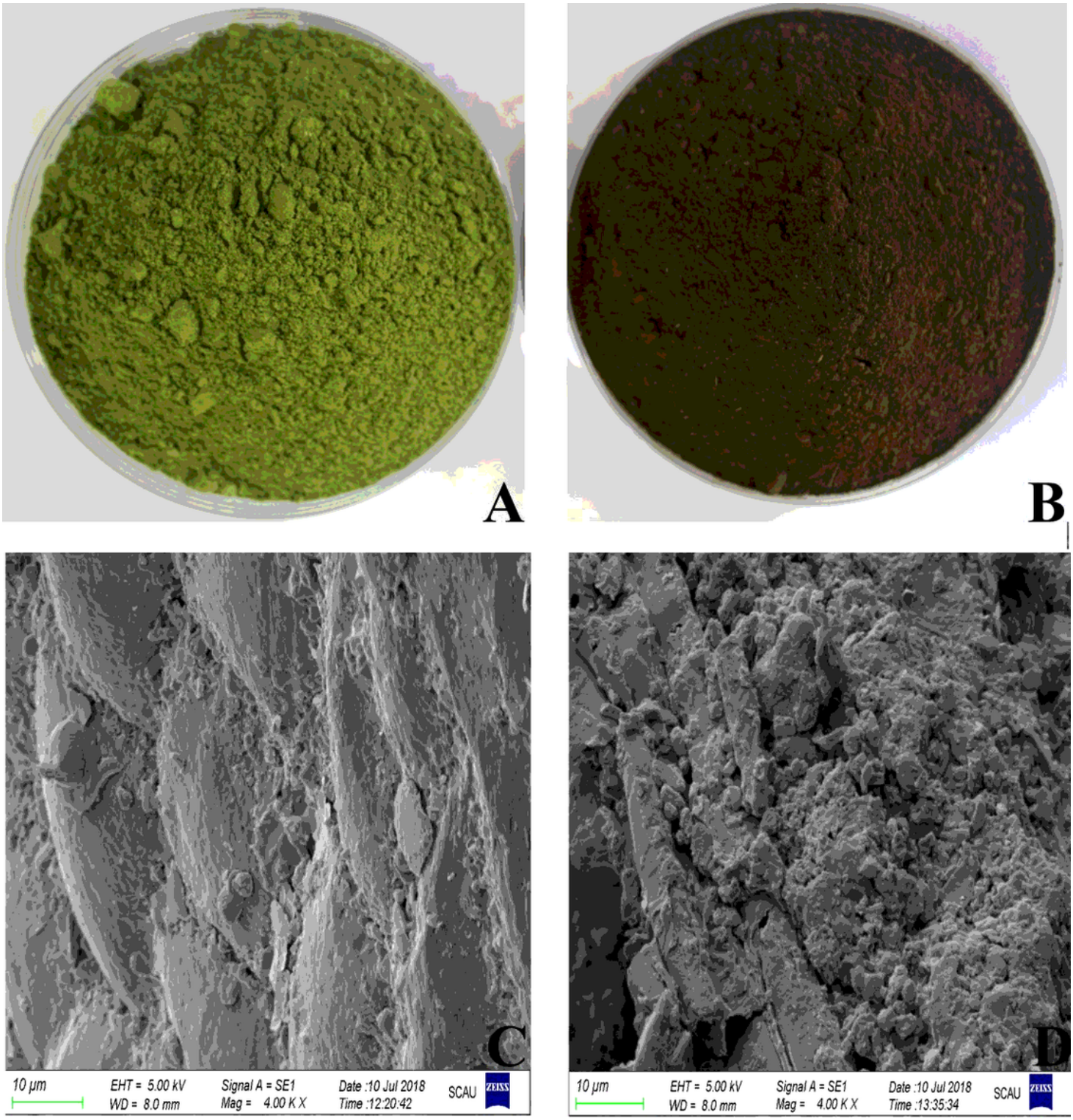
Table 2 Amino acid composition of FDLF and UDLF.

| Amino acid composition, % | UDLF | FDLF |
|---|---|---|
| Arg | 1.42±0.02[b] | 1.60±0.01[a] |
| His | 0.50±0.02[b] | 0.58±0.01[a] |
| Ile | 0.97±0.06[b] | 1.15±0.03[a] |
| Leu | 2.06±0.05[b] | 2.38±0.04[a] |
| Lys | 1.40±0.10 | 1.35±0.02 |
| Phe | 1.31±0.06[b] | 1.53±0.01[a] |
| Thr | 1.12±0.05[b] | 1.38±0.02[a] |
| Val | 1.23±0.08[b] | 1.47±0.01[a] |
| Trp | 0.65±0.07[b] | 1.03±0.11[a] |
| Ala | 1.49±0.06[b] | 1.80±0.02[a] |
| Asp | 2.13±0.11[b] | 2.48±0.10[a] |
| Cys | 0.11±0.04[b] | 0.15±0.02[a] |
| Glu | 2.80±0.08 | 2.69±0.20 |
| Gly | 1.21±0.01[b] | 1.55±0.05[a] |
| Pro | 1.01±0.04[b] | 1.44±0.01[a] |
| Ser | 1.07±0.04[b] | 1.27±0.04[a] |
| Tyr | 0.84±0.01[b] | 1.03±0.01[a] |
| Indispensable AA, % | 10.66±0.51[b] | 12.47±0.26[a] |
| Dispensable AA, % | 10.65±0.24[b] | 12.41±0.20[a] |
| Total AA | 21.31±0.75[b] | 24.88±0.46[a] |

Table 3 Correlation analysis of the concentrations of total phenols and flavones and antioxidant activity in FDLF.

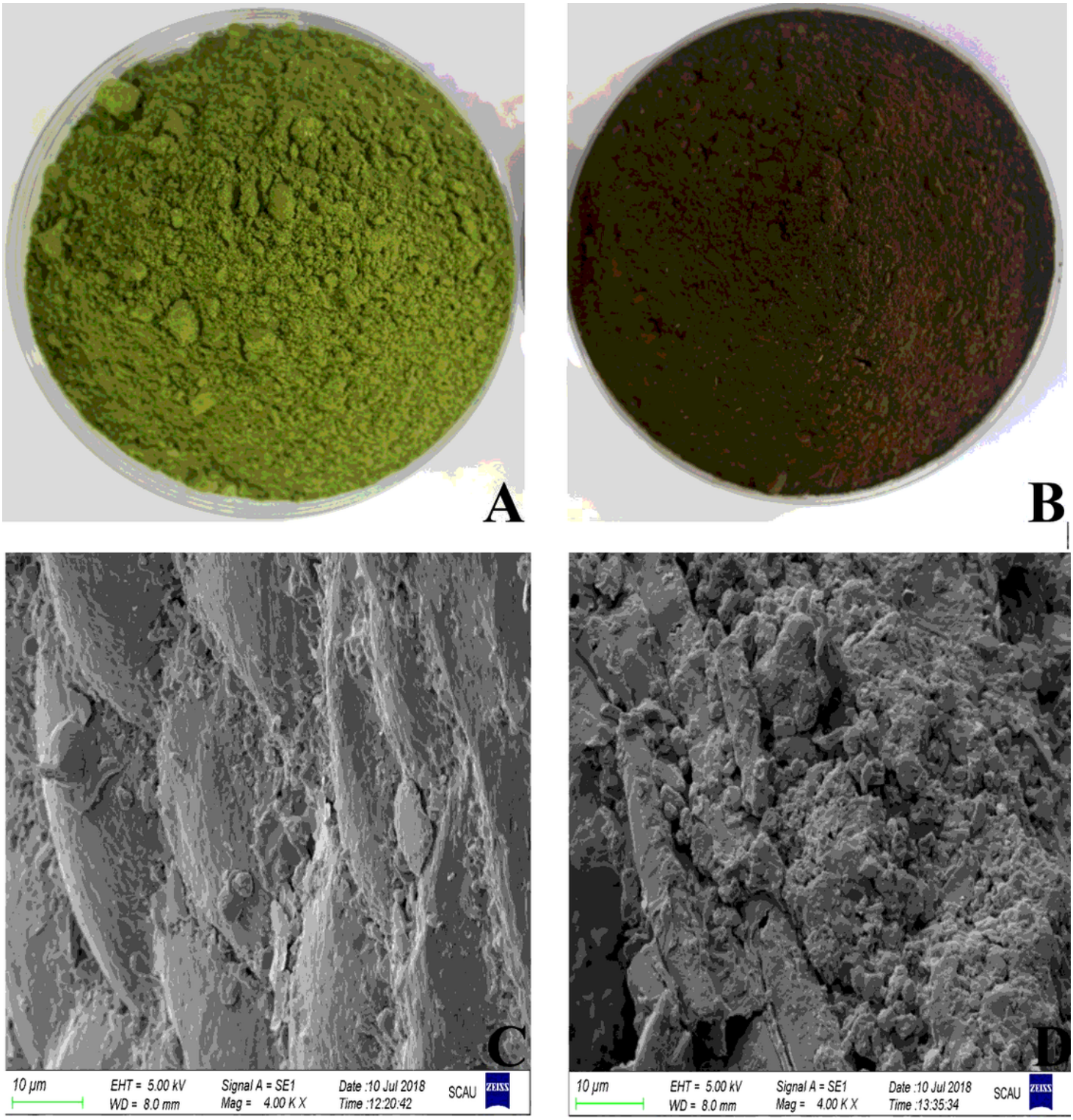
| Item | DPPH free radical scavenging rate | $ABTS^+$ free radical scavenging rate | Total antioxidant ability |
|---|---|---|---|
| total phenols | 0.790* | 0.856** | 0.932** |
| flavones | 0.975** | 0.571 | 0.855** |

# Figures



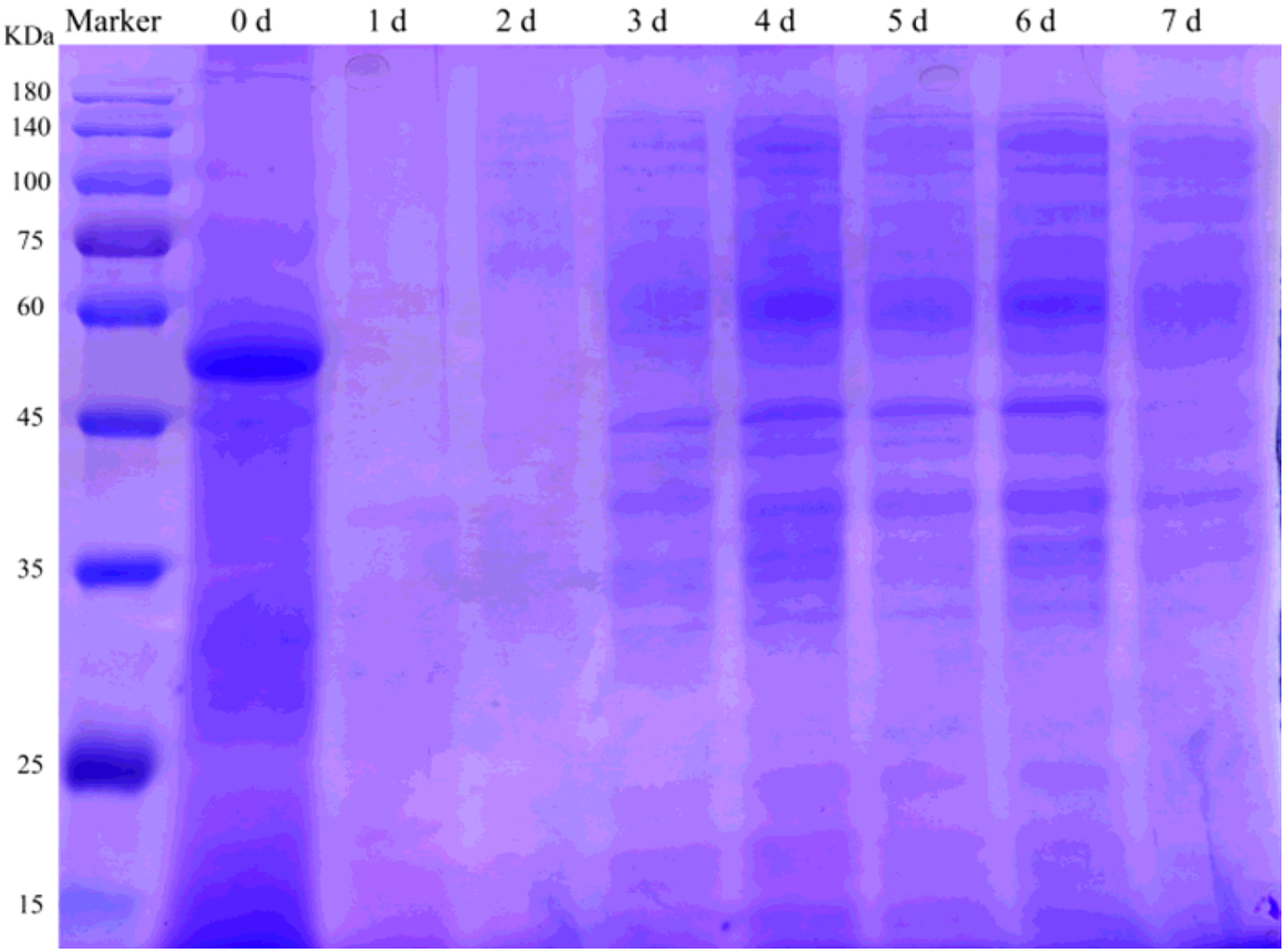

## Figure 1

Phenotype and scanning electron microscope images (×4000) of UDLF (A, C) and FDLF (B, D).



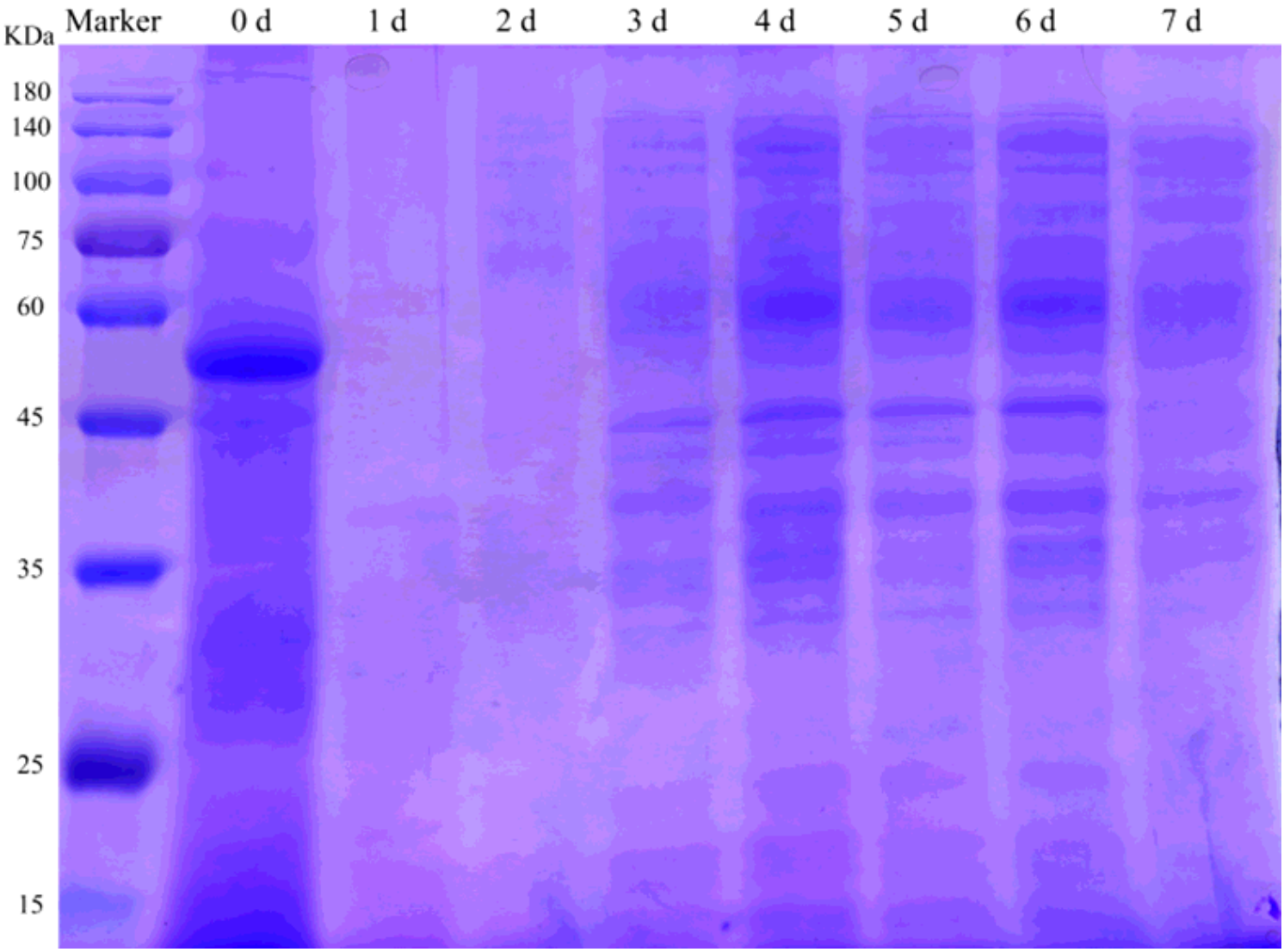

## Figure 1

Phenotype and scanning electron microscope images (×4000) of UDLF (A, C) and FDLF (B, D).



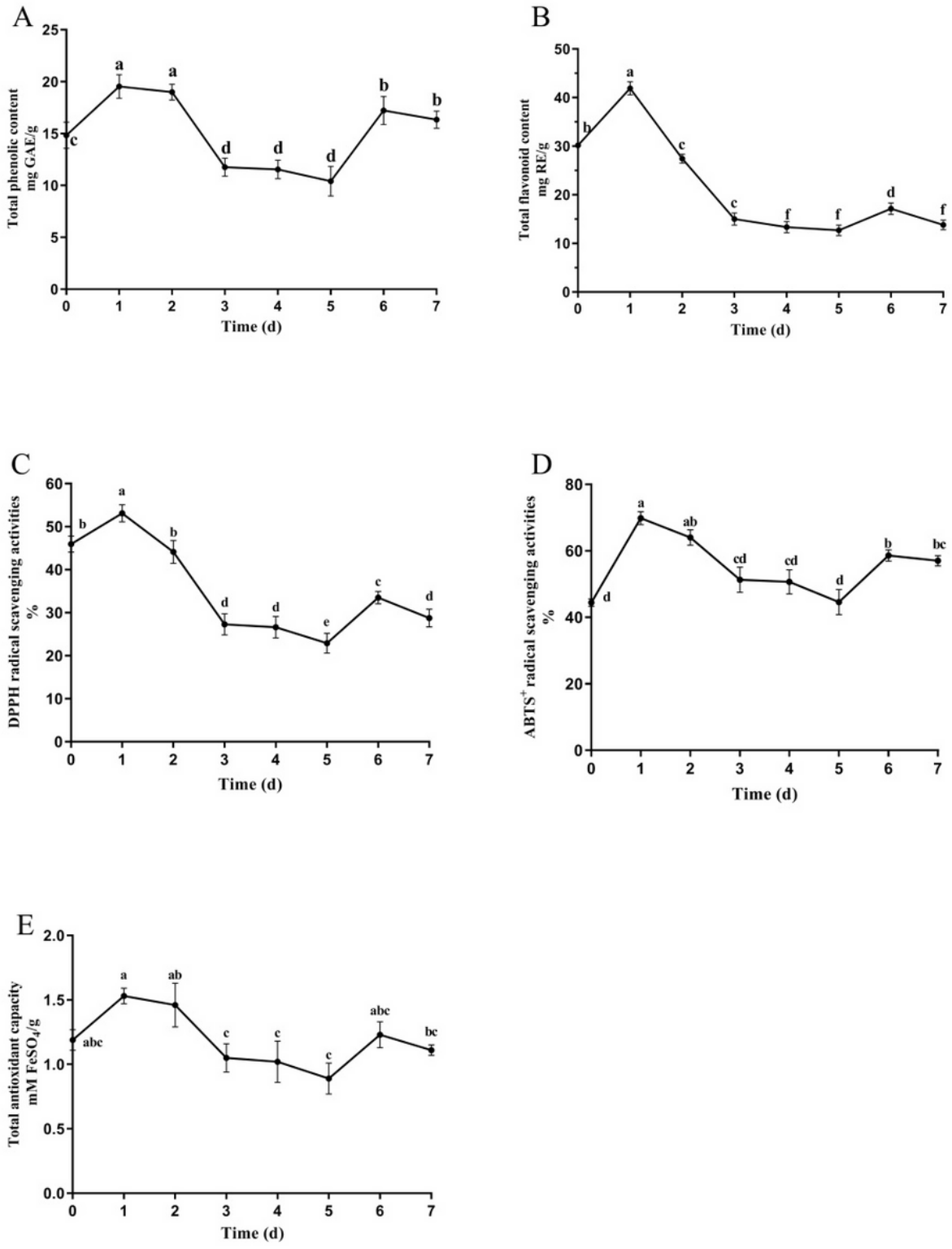

## Figure 2

SDS-PAGE analysis of protein profiles in DLF during fermentation.



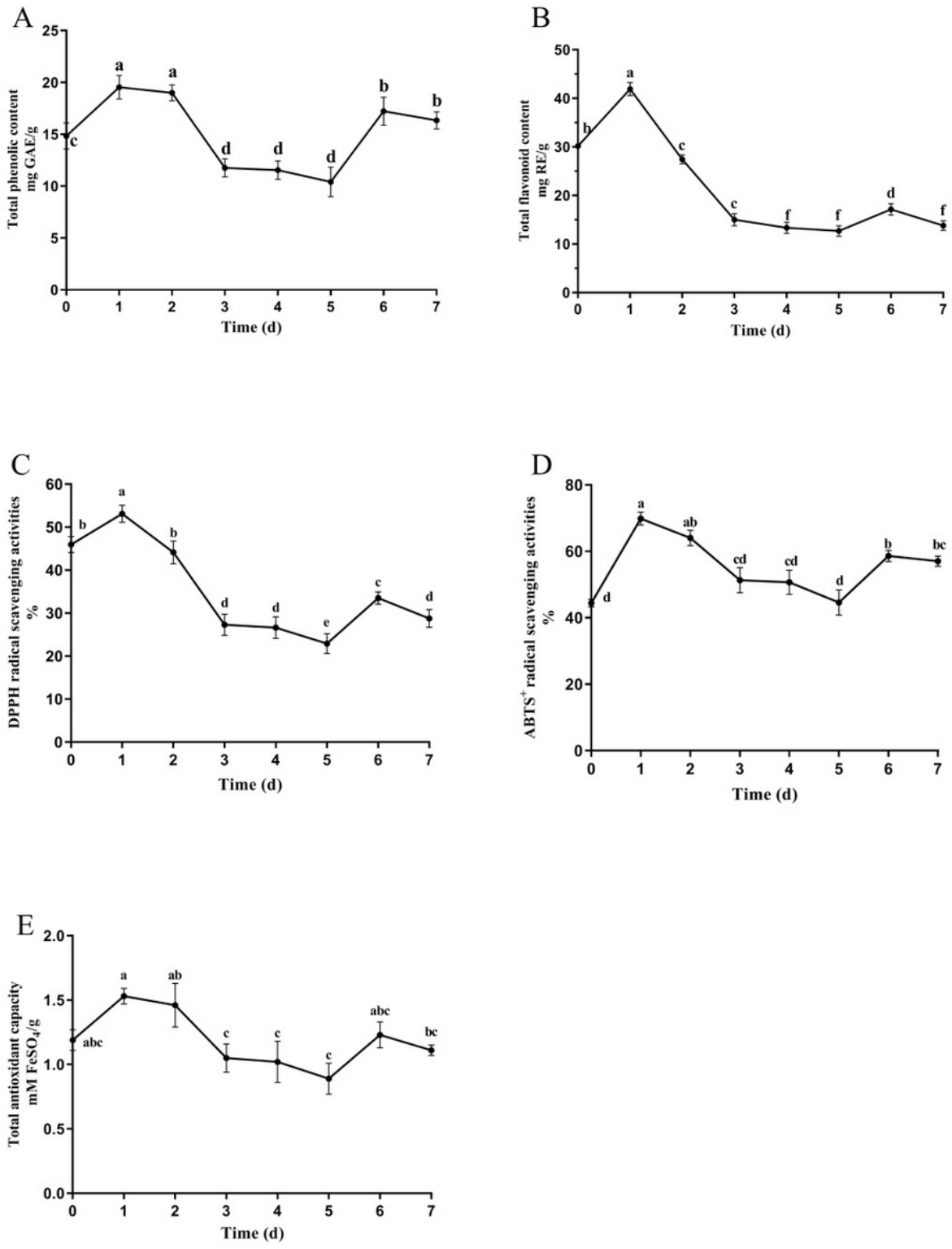

## Figure 2

SDS-PAGE analysis of protein profiles in DLF during fermentation.




## Figure 3

Dynamic changes in total flavonoid, phenolic content and antioxidant capacity.




## Figure 3

Dynamic changes in total flavonoid, phenolic content and antioxidant capacity.