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Nutrition Component Adjustment of Distilled Dried Grain with Solubles via *Aspergillus niger* and Its Change about Dynamic Physiological Metabolism

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Abstract: The low fiber digestibility and unbalanced amino acids restricted the use of DDGS in swine diets. Key nutrition components dynamic monitoring and key regulatory pathways analysis were performed to find the rules of nutrition changes for DDGS fermented by *Aspergillus niger*. Cellulose and hemicellulose were reduced to 15.3% and 15.2%. 1,4-D-Xylobiose was decreased from 16.8 μg/mL to 0.2 μg/mL. Lys, Arg, and Thr were increased to 3.00%, 2.89%, and 4.40%, and met the requirements of pigs. The whole fermentation process was divided into three stages. Cellulose degradation and Lys and Arg synthesis occurred in the early stage, while Asp synthesis occurred in the last stage. α-Ketoglutarate was the key factor for *Aspergillus niger* degrading cellulose to synthesize Lys and Arg. The key active metabolic pathways that respond to the changes in nutrition were identified which preliminarily revealed the rules of nutrition adjustment of DDGS during fermentation with *Aspergillus niger*.

Keywords: DDGS; *Aspergillus niger*; fermentation; nutrition adjustment; regulatory pathways

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1. Introduction

In the corn-ethanol dry milling industry, one ton of ethanol is produced from three tons of corn, with 0.92–0.95 tons of distillers dried grains with solubles (DDGS) as the by-product [1]. The annual global production of DDGS is projected to increase to about 120 million tons by 2024 [2]. DDGS is rich in a variety of nutrients needed by animals, including a large amount of protein, lipids, minerals, and vitamins. It was considered a replacement for corn in animal diets, which provides both energy and protein to the ration matrix. However, the inclusion rate of DDGS in swine diets is restricted because of the lower fiber digestibility and unbalanced amino acids. The fiber is resistant to digestion by enzymes in the small intestine [3]. The lower digestibility of fiber in DDGS results in an increased quantity of manurle being excreted from swine who are fed these products [4]. Lysine (Lys) is the first limiting amino acid in swine diets, and the content of Lys in DDGS is relatively low compared with the need for growth and development of swine. Excessive leucine (Leu) relative to Lys interferes with the utilization of isoleucine (Ile) and valine (Val), which may reduce feed intake and growth rate in swine [5,6]. Therefore, improving key amino acids and reducing fiber in DDGS has the potential to raise its feeding value for swine and increase the benefits of corn-ethanol refineries.

Fermentation is one of the most promising ways to improve the nutritive quality of DDGS [7]. *Aspergillus niger (A. niger)* is one of the most important microorganisms used in biotechnology. *A. niger* has the ability to use a wide range of sugars, including

glucose, maltose, xylan, xylose, and lactose. *A. niger* also produces enzymes, such as acetylesterase, amylase, pectinases, fucosidase, glucose oxidase, glucosidase, mannanase, phospholipase, phytase, prolyl endopeptidase, triacylglycerol lipase, trehalase, cellulase, and xylanase [8–10]. *A. niger* enzymes are generally regarded as safe (GRAS) by the United States Food and Drug Administration (USFDA) and are commonly used in food processing [11,12]. These enzymes could change the chemical profiles of DDGS. Using *A. niger* in fermentation has been reported to degrade cellulose and improve protein [13–17]. Moreover, *A. niger* produces lactic acid that has potential as a favorable animal feed [18]. It is feasible to utilize *A. niger* to upgrade the nutritional value in DDGS via solid-state fermentation, which could have the potential to reduce co-products processing cost.

Understanding the dynamic changes of key nutrition components and metabolic pathways during fermentation with *A. niger* plays a major role in the nutrition adjustment of DDGS. Although some previous studies have attempted to improve the nutritional value of DDGS via fermentation with different microorganisms, the utilization of *A. niger* to improve the feeding value of DDGS seems to be largely uncharted, and there is no comprehensive analysis of the metabolite succession process during fermentation or how to control the fermentation process for the targeted accumulation of nutrients [19–21]. Untargeted metabolomics provides comprehensive insights into complex metabolomes and allows for the discovery of novel biomarkers and generating new metabolic hypotheses [22], and may unravel the nutritional aspects of fermented feed.

In this study, the dynamic changes of key nutrition components during fermentation of DDGS with *A. niger* were monitored. The rules of the conversion and improvement of the nutrient composition of DDGS were analyzed. Moreover, the key regulatory pathway was proposed based on this. These results preliminarily revealed the mechanism of nutrition changes for DDGS fermented with *A. niger*, and it provided basic data for directional adjustment of DDGS feed nutrients by *A. niger* fermentation in the absence of starch-carbon sources.

2. Materials and Methods

2.1. Preparation of Microorganism and Substrate

2.1.1. Microorganism, Medium, and Culture

A. niger (LCCC 30016) was propagated on agar plates containing 3% malt extract and 1.5% agar. The plates were incubated at 30 °C for 3–4 days until complete sporulation. The spores from the plates were dislodged with sterile water containing glass beads. The suspensions were stored at −80 °C in spore suspensions frozen in 25% glycerol.

2.1.2. Substrate and Inoculum

DDGS was obtained from Weifang yingxuan industrial Co., Ltd. (Weifang, China), with a starch content of approximately 1.02%. For inoculum preparation, *A. niger* was first activated by culture in a liquid medium containing 3% malt extract at 30 °C and 150 rpm for 24 h. The activated culture (population of 1.05×10^8 CFU/mL) was used as the inoculum for the fermentation of DDGS.

2.2. Solid-State Fermentation

We have optimized fermentation temperature (25–35 °C), inoculum amount (5–20%), and material-to-water ratio (1:1–1:4). The fermentation conditions of DDGS fermented by *A. niger* were based on the previous optimization experiments. In total, 20 g DDGS and 40 g water were mixed and inoculated with 10% (*v*/*w*) of *A. niger*. The substrate was fermented at 30 °C for 7 days. The samples were collected every day. After fermentation, the samples were treated at 105 °C for 20 min to stop fermentation. No nutrients or minerals were supplemented to the substrates in this study [23,24]. The fermentation process was repeated 6 times.

2.3. Nutrient Composition Analysis

2.3.1. Determination of Nutrient Content

The content of crude protein was determined using the Kjeldahl method [25]. In total, 0.20 g fermented sample was digested with mixed catalyst and 10 mL sulfuric acid at 380 °C for 2.5 h, then distilled by addition of 10 mol/L sodium hydroxide solution using a semi-automatic Kjeldahl appearance. The volatile ammonia from distillation was absorbed with 20 mL of 20 g/L boric acid, and finally titrated with 0.1 mol/L hydrochloric acid.

The concentration of total sugar was measured by the spectrophotometric method [26]. In short, 1.00 g of the sample, 25 mL distilled water, and 10 mL 12 mol/L hydrochloric acid were added into a 250 mL conical flask, shaked well until a homogeneous suspension was formed and then hydrolyzed in a boiling water bath for 60 min. After cooling the hydrolysate, the filter residue was filtered and washed with distilled water, then the filtrate and the washing solution were diluted to 50 mL. In total, 1 mL sample, 1 mL phenol solution, and 5 mL sulfuric acid were added into a 10 mL colorimetric tube, where it stood for 10 min, and the reaction solution was shaken by vortex and cooled to room temperature. Finally, it was measured the absorbance at a 490 nm wavelength.

The crude fat content was determined according to Shin et al.'s method [27]. Approximately 2.00 g sample, 8 mL distilled water, and 10 mL 12 mol/L hydrochloric acid were added into a 50 mL test tube, then placed in a shaking water bath at 70–80 °C, stirred once every 5–10 min, and maintained for 40 min. After the sample had cooled to room temperature, 10 mL ethyl alcohol and absolute ether were added and shaken. The solution was allowed to stand for 10–20 min until the top and bottom layers were separated. The ether (top) layer was collected and evaporated in a water bath, and finally dried and weighed.

The content of ash was determined according to the AOAC (1995). Ash was quantified after calcination in a muffle furnace at 550 °C for 3 h.

2.3.2. Determination of Amino Acids Composition

The analysis of amino acid concentration was performed by an Amino Acid Analyzer (L-8900, HITACHI, Tokyo, Japan) fitted with a Hitachi high-performance cation-exchange column with 57 °C column temperature [28]. Briefly, 0.012 g sample power was fully dissolved in 3 mL of 6 mol/L HCl and hydrolyzed at 110 °C for 23 h. In total, 1 mL of hydrolysate was diluted to 25 mL, then 1 mL was freeze-dried. The reconstitution of samples used 1 mL 0.02 mol/L HCl, then filtered by a 0.22 μm syringe filter. The concentration of amino acid was quantified by the external standard method.

2.3.3. Determination of Lignocellulose Composition

The analysis of lignocellulose composition was determined according to the determination of structural carbohydrates and lignin in biomass [29]. 1.0 g sample was fully immersed in 30 mL of deionized water for 30 min then dried at 105 °C. Dried sample power was hydrolyzed with 72% sulfuric acid at 30 °C for 60 min, then diluted the sulfuric acid concentration to 4% with deionized water and hydrolyzed under 121 °C high pressure for 60 min. The supernatant was filtered, adjusted to a pH of 6.0–8.0, and filtered by using a 0.22 μm syringe filter.

The determination of glucose, xylose, arabinose, and cellobiose was performed by high-performance liquid chromatography (HPLC) (ACCHROM S6000) system. The measurement conditions were as follows: ROA Organic Acid column (300 × 7.8 mm) (Bio-Rad, Hercules, CA, USA, Aminex HPX-87H), column temperature at 60 °C, the mobile phase was 0.01 mol/L H_2SO_4, 20 μL of injection volume, and the flow rate was set to be 0.40 mL/min.

2.4. Metabolomics Experiments

Samples were dissolved in water and then homogenized at 2800 r/min for 2 min, then cold methanol was added and vortexed for 2 min. Cold methyl tert-butyl ether was added and vortexed for 2 min, then the samples were centrifuged at 4 °C, 8000 rpm for 10 min.

The nonpolar solution of samples was collected and filtered through 0.22 μm syringe filters to be tested.

Metabolites in fermented DDGS were subjected to metabolomics analysis using a Thermo QE HF-X mass spectrometer with heat electrospray ionization (HESI) using a Dionex UltiMate 3000 system (Dionex Softron GmbH, Germering, Germany) with an Acquity UPLC BEH HILIC column (1.7 μm × 2.1 mm × 150 mm; Waters Corporation, Milford, MA, USA) [30]. The mobile phase A was 10 mmol/L ammonium formate and 0.125% formic acid (*v*/*v*) in water, while the mobile phase B was acetonitrile: water (95:5, *v*/*v*) containing 10 mmol/L ammonium formate and 0.125% formic acid (*v*/*v*). Samples of an injection volume of 1 μL were analyzed with a fixed flow rate of 0.4 mL/min. Spectra acquisition was performed in positive and negative ionization modes, and spectra were acquired over a mass range of *m*/*z* 50–1500. In both modes, the sheath gas flow rate was 60 arb and the aux gas flow rate was 20 arb. The spray voltage was at 3.5 kV and the ion transport tube temperature was 380 °C. Four analytical replicates from each sample were analyzed in QE HF-X.

The measured data were regressed and corrected by internal standards. The compounds detected were identified via Compound Discoverer 3.0. The primary mass spectrum and secondary mass spectrum compared the correct substances as accurately identified metabolites.

2.5. Data and Statistical Analysis

MetaboAnalyst 5.0 [31] was used for regression analysis, partial least squares discriminant analysis (PLS-DA), VIP scores, volcano plot, metabolomic pathway, etc. Boxplots were drawn using XLSTAT 2019. SPSS was used to analyze the significant difference between different samples. The final results were shown as the mean value of more than three replicates.

3. Results and Discussion

3.1. Changes in Main Nutrients of DDGS during Fermentation

Based on the optimal fermentation conditions of DDGS fermented by *A. niger*, the changes in main nutrients in the whole fermentation process were analyzed to further observe the characteristics of nutritional transformation. The total content changes of protein, fat, carbohydrate, and ash in DDGS during fermentation with *A. niger* were analyzed. As shown in Figure 1, *A. niger* significantly changed the content of major nutrients in DDGS. At the beginning of fermentation, *A. niger* consumed part of the protein in DDGS as it grew. The total content of protein in DDGS significantly decreased within the first day of fermentation. From the second day, the protein content in DDGS gradually increased until the end of fermentation. It indicated that *A. niger* consumed the protein in DDGS at a slower rate than the protein it produced itself. Compared with unfermented DDGS, the protein in DDGS at the end of fermentation increased from 9.5% to 11%. After fermentation by *A. niger*, the improved crude protein concentration could be due to the degradation of complex fiber forms with the protein, with no extraneous nitrogen source added [32]. Similar protein improvement by fermentation was also reported on fermented wet distiller grains with solubles by *Rhizopus oryzae* [32].

Total sugar content in DDGS during fermentation appeared a peak around the second day of fermentation. From the fourth day onwards, its level remained constant. Compared with unfermented DDGS, the total sugar content of DDGS at the end of fermentation increased by 3.9%. In the determination of total sugar, only the content of soluble carbohydrates was counted, while the content of insoluble carbohydrates, such as cellulose and hemicellulose, was not included. Most of the carbohydrates in DDGS were fiber which was not easy to digest and absorb. This indicated that *A. niger* produced a large amount of cellulase to degrade cellulose, hemicellulose, and other substances in DDGS into soluble sugars at the beginning of fermentation [9]. However, with the progress of fermentation, *A. niger* would further utilize the newly generated high-quality soluble carbon source

for life activities. This contributed to the phenomenon that the total sugar content peaks between the second and third days of fermentation and then falls back and stabilization after the fourth day of fermentation. It also suggested that *A. niger* changed the fiber content in DDGS which was difficult to digest and absorb.

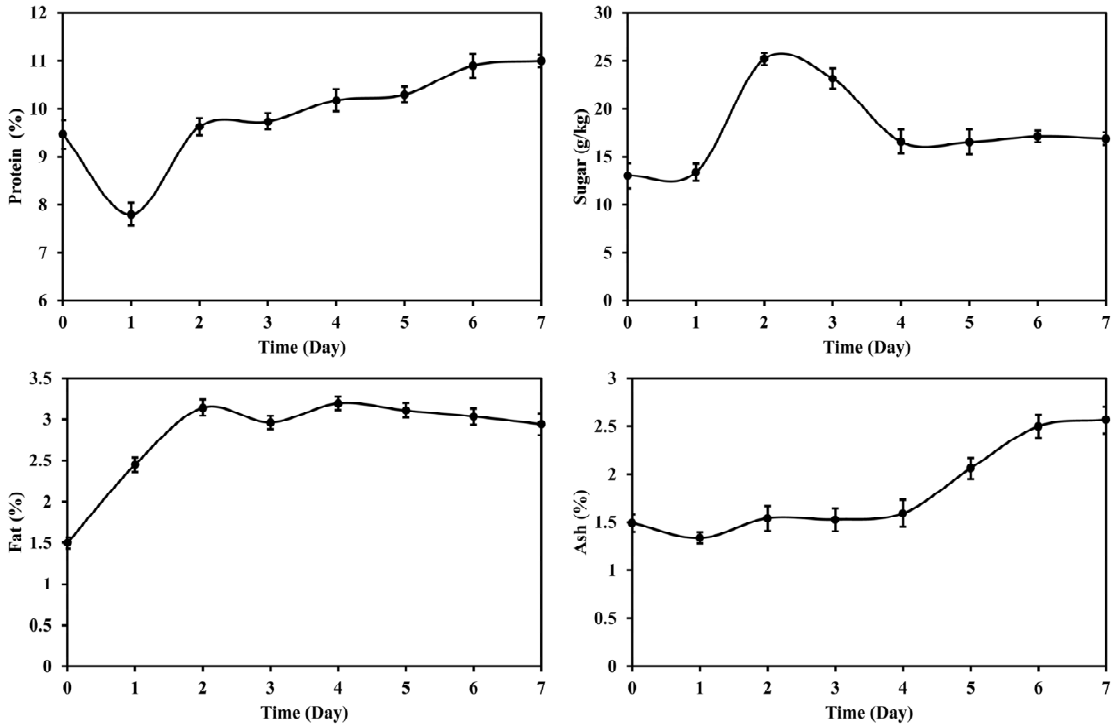

Figure 1. Main nutrient content changes of DDGS during fermentation with *Aspergillus niger*. Note: The samples used for analysis were wet samples.

The fat and ash contents of DDGS fermented by *A. niger* also increased. Fat content increased mainly in the first two days of fermentation. Ash content did not increase until the fourth day of fermentation. After fermentation, the fat and ash contents of DDGS increased by 1.5% and 1%, respectively. All of these indicated that *A. niger* had a good potential to modify DDGS nutrition. The changes in specific nutrients were further analyzed, especially the amino acid composition and cellulose content, which were the main factors that restricted DDGS used in pig feed.

3.2. Effects of A. niger on Degradation of Structural Carbohydrates in DDGS

The production of enzymes by *A. niger*, such as cellulase, xylanase, and amylase, may cause the degradation of the fiber components in DDGS. Thus, the percentages of cellulose and hemicellulose were detected in this study. As shown in Figure 2, significant differences were observed between the fermented and unfermented DDGS. Compared with unfermented DDGS, the fermented DDGS contained less cellulose and hemicellulose. It indicated that cellulose and hemicellulose in DDGS were degraded into oligosaccharides, monosaccharides, or disaccharides as the fermentation progressed. After fermentation by *A. niger*, the percentage of cellulose and hemicellulose in DDGS decreased to 15.3 $\pm$ 0.01% and 15.2 $\pm$ 0.10%, respectively. The amount of degradation both exceeded 3%, which was higher than the degradation rate (by 2.15%) of corn DDGS fermented by *Bacillus subtilis* and *Lactobacillus plantarum* [19]. The degradation of cellulose and hemicellulose directly or indirectly produced monosaccharides, disaccharides, or trisaccharides that were the main energy sources in swine diets [33].

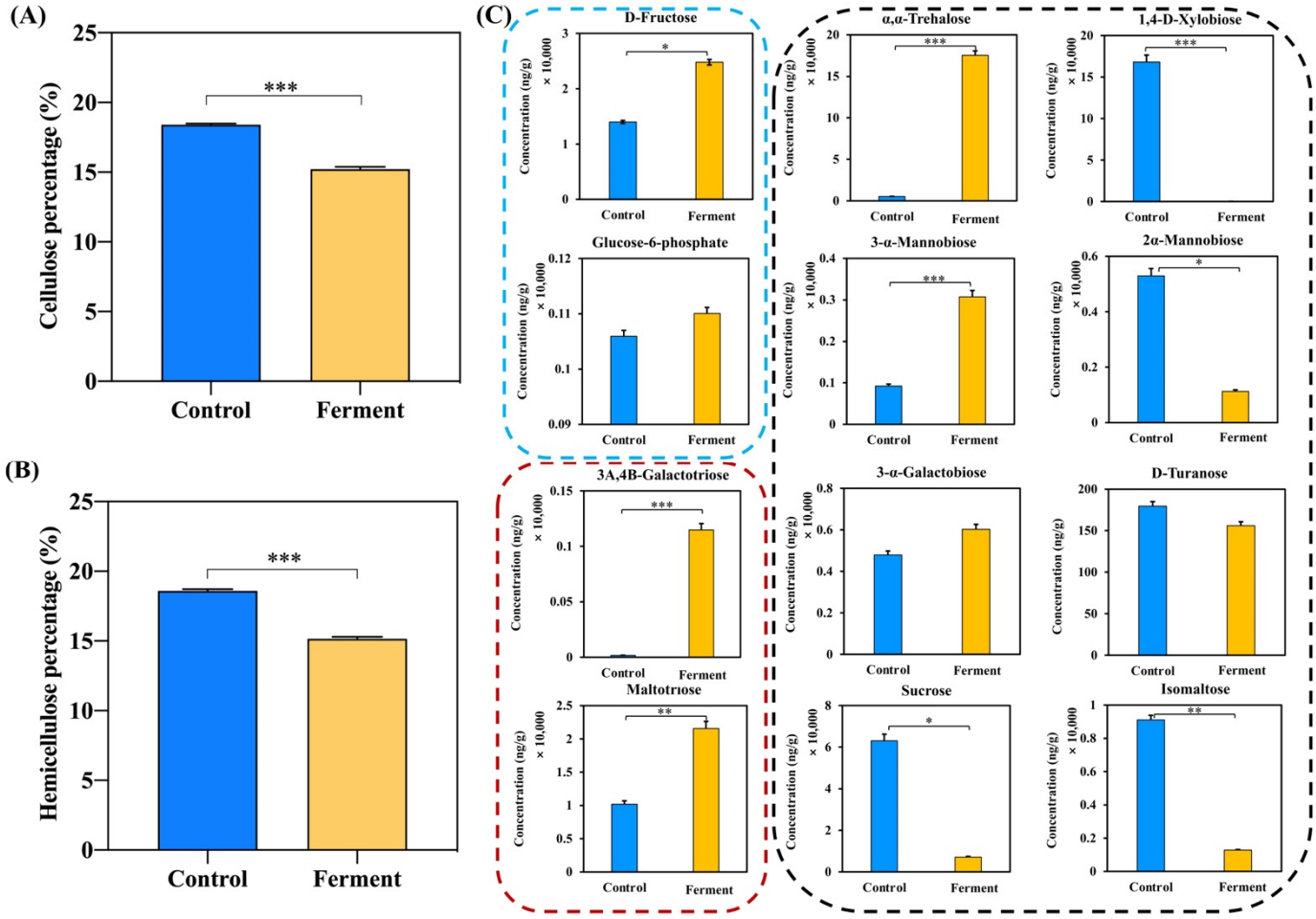

Figure 2. The content of hemicellulose, cellulose, monosaccharides, disaccharides, and trisaccharides in DDGS before and after fermentation with *Aspergillus niger*. (**A**) The change of cellulose content; (**B**) The change of hemicellulose content; (**C**) The change of monosaccharides, disaccharides, and trisaccharides. Monosaccharides in the blue box; disaccharides in the black box; trisaccharides in the red box. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.

In terms of the monosaccharides, disaccharides, or trisaccharides that may be produced after fermentation, a total of 12 types of sugars, α,α-Trehalose, 1,4-D-Xylobiose, 2α-Mannobiose, 3A,4B-Galactotriose, 3α-Galactobiose, 3α-Mannobiose, D-Fructose, Glucose-6-phosphate, Isomaltose, Maltotriose, Sucrose, and D-Turanose were detected. After fermentation, the contents of α,α-Trehalose, 3A,4B-Galactotriose, 3α-Mannobiose, and D-Fructose in DDGS increased significantly, while the contents of 1,4-D-Xylobiose, Sucrose, 2α-Mannobiose, and Isomaltose decreased significantly. Although the contents of 3α-Galactobios and Glucose-6-phosphate also increased after fermentation, the increase was not significant. The contents of trehalose and xylose varied the most before and after fermentation. After fermentation, the concentration of α,α-Trehalose increased from 5.3 μg/mL to 17.5 μg/mL, while the concentration of 1,4-D-Xylobiose decreased from 16.8 μg/mL to 0.2 μg/mL. The large amount of xylose in DDGS made it unsuitable for pig digestion and absorption [34]. Xylose in DDGS could be converted into other nutrients or other sugars after fermentation, which was helpful for DDGS application in pig feed. It indicated that the nutritional modification of DDGS by *A. niger* better met the nutritional intake requirements of pigs.

3.3. Effects of *A. niger* on Amino Acid Composition Proportion Adjustment

Protein is the most important nutrient in pig feed. Its amino acid composition directly affects the nutrient absorption of pigs. To use DDGS as pig feed, its amino acid composition is one of the necessary evaluation indexes. As shown in Figure 3, the composition ratios of 16 amino acids, including essential and non-essential amino acids for pig growing, in fermented and unfermented DDGS were analyzed. In terms of essential amino acids for pig in DDGS, proportions of Lys, threonine (Thr), phenylalanine (Phe), and arginine (Arg) to total amino acids content were increased significantly after fermentation, while that of Leu, Val, proline (Pro), and methionine (Met) decreased significantly. The proportions of tyrosine (Tyr), aspartic acid (Asp), and glycine (Gly), non-essential amino acids to pig, also increased significantly. In terms of DDGS, the concentrations of Val and Leu are in excess, while Lys and Thr are deficient relative to the requirements of pigs [35]. Furthermore, Met had been considered the most toxic amino acid if fed excessively in pig diets [36]. Therefore, *A. niger* adjusted the amino acid composition of DDGS for application in pig feed. As shown in Figure 3, the proportions of Lys, Arg, and Thr to total amino acids in fermented DDGS were 3.00%, 2.89%, and 4.40%, which were increased by 50.0%, 660%, and 37.1%, respectively. It was recommended that the Lys concentration of corn DDGS used in pig diets should not be less than 2.80% expressed as a percentage of crude protein [33]. The DDGS fermented with *A. niger* satisfies the requirements to be used in pig feed. The percentage of Leu in fermented DDGS was 5.65%, which was decreased by 32.6% in unfermented DDGS. This indicated that the DDGS fermented by *A. niger* contributed to the balance of amino acids.

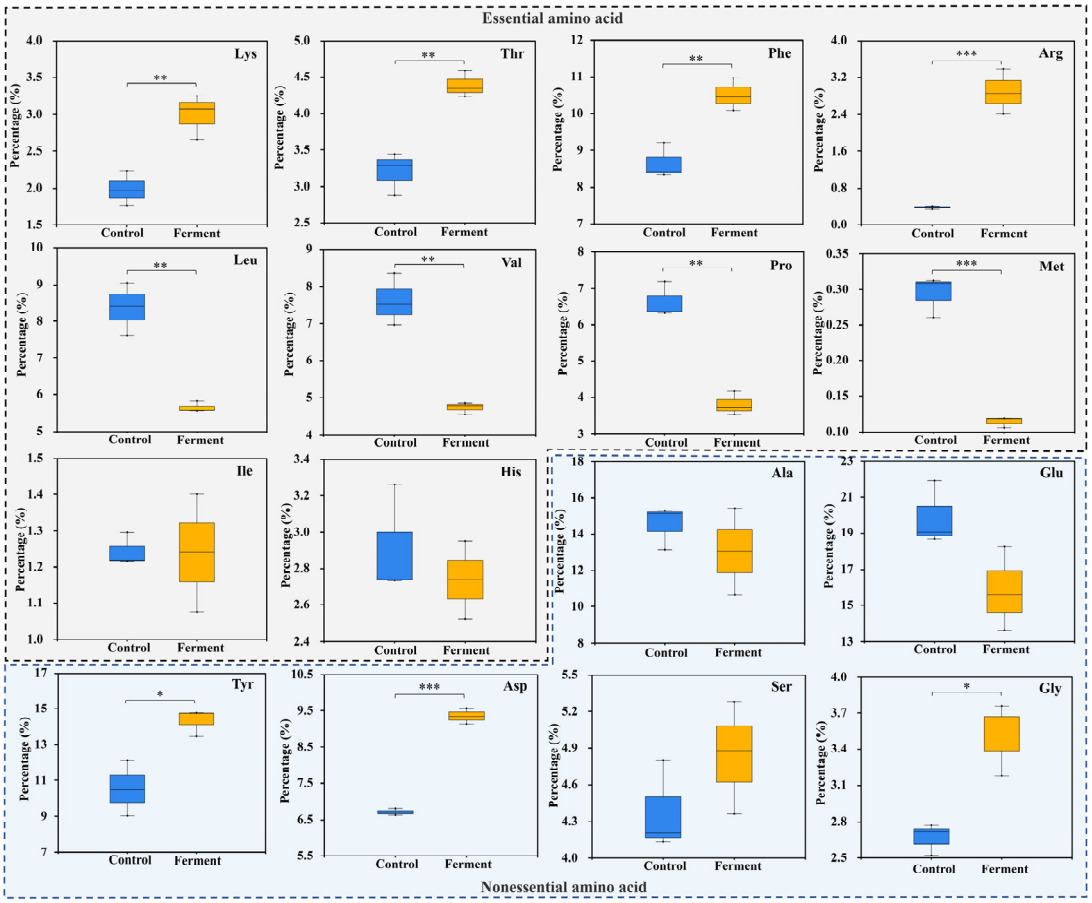

Figure 3. Different amino acid composition ratio of DDGS before and after fermentation with *Aspergillus niger*. The amino acids in the gray area are essential amino acids for pig growth while the non-essential amino acids in the blue area. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.

In order to trace how *A. niger* changed the amino acid composition, possible amino acids and short peptides produced during fermentation were detected and analyzed. As shown in Figure 4, 128 amino acids and short peptides were detected in DDGS. In total, 45 key amino acids and short peptides that led to the significant differences were screened. The first day of fermentation might be an important stage for changes in Arg and Lys content in DDGS. Most of the newly produced short peptides or amino acids were related to Arg and Lys. According to the heat map, the distribution of various short peptides and amino acids on the second day of fermentation was unique. This indicated that the second day of fermentation might be an important transitional stage of DDGS fermentation by *A. niger*. From the third day to the fifth day of fermentation, the changes in amino acids or short peptides were similar. It might be the stable stage of DDGS nutritional adjustment by *A. niger*. In the last stage of fermentation, the sixth and seventh days of fermentation, the main period of Asp content changed. It indicated that DDGS nutrition changed in a periodic manner. Directional shaping of nutrient composition can be realized through stage control. Analysis of key metabolic pathways during DDGS fermentation by *A. niger* will help to achieve directional shaping of nutrient composition.

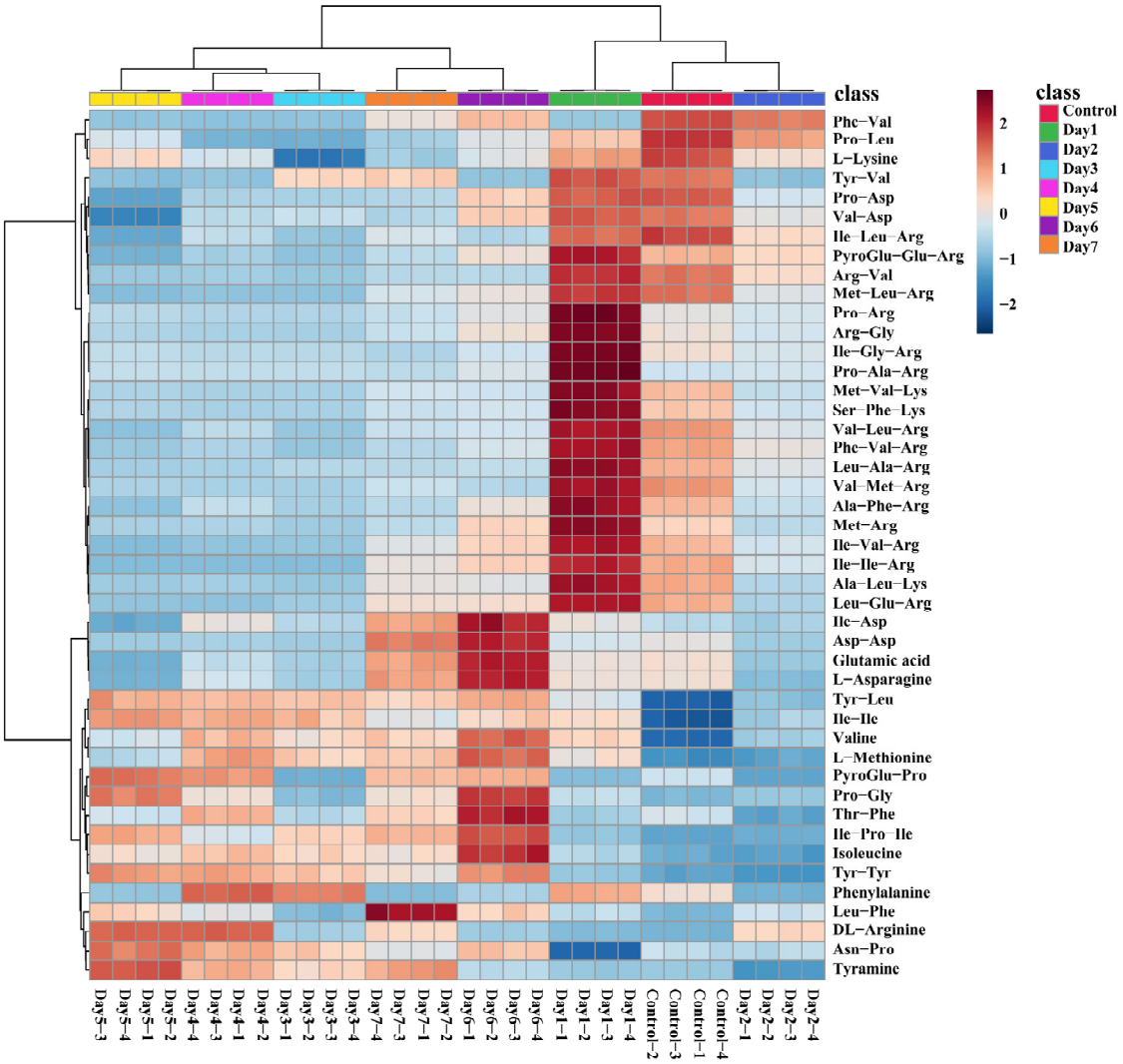

Figure 4. The heatmap of main amino acids and short peptides produced by *Aspergillus niger* during fermenting DDGS. The compounds shown in the heatmap were screened with VIP > 1.0.

3.4. Analysis of Key Regulatory Pathways of A. niger for Adjusting DDGS Nutrition

Dynamic changes of metabolites during DDGS fermentation by *A. niger* were observed. As shown in Figure 5, the metabolites related to the nutrition changes above were compared by comprehensive statistical analysis methods. As shown in Figure 5A, the fermentation process could be divided into three different stages by time. C-DDGS, D1-DDGS, and D2-DDGS were classified into one category. However, the second day of fermentation and the first day of fermentation were classified into the same stage, and there were differences between them according to the PLS-DA score chart. The second day of fermentation might be the critical transition period between the first and second stages of fermentation. This was consistent with the previous process of nutrient change. The nutrient composition of DDGS after fermentation was significantly different from that of unfermented DDGS from the third day. The results of PLS-DA, D3-DDGS, D4-DDGS, and D5-DDGS could be classified into one category, while the sixth and seventh days of fermentation could be classified into the end stage of fermentation. Based on this phenomenon, fermentation could be regulated by adding the nutrients needed for *A. niger* at this stage. Therefore, the metabolites of *A. niger* were further analyzed and possible regulatory pathways were explored.

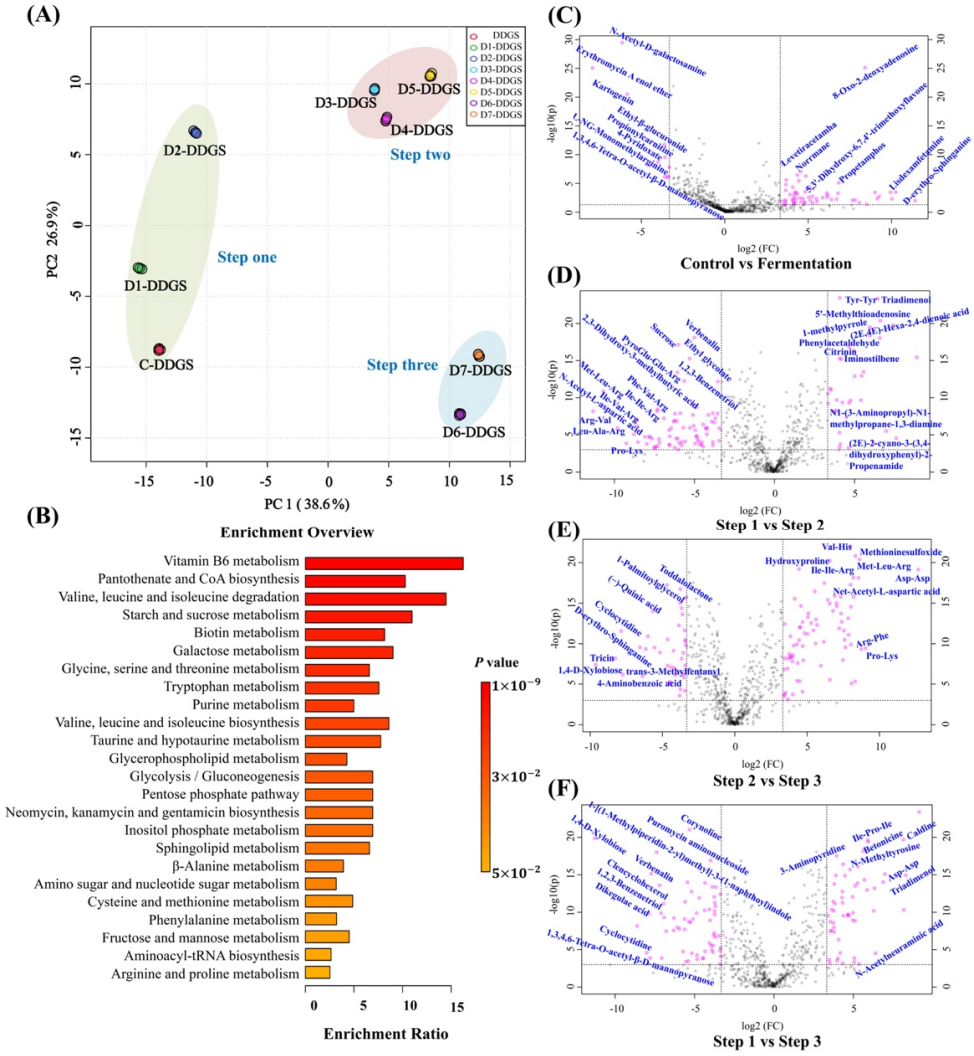

Figure 5. Statistical analysis of metabolites produced in DDGS fermentation process with *Aspergillus niger*. (**A**) PLS-DA score plot for the fermentation process; (**B**) Enrichment overview of metabolic pathways; (**C**) Volcano plot of metabolites in DDGS before and after fermentation; (**D**) Volcano plot of metabolites in DDGS of step 1 and step 2; (**E**) Volcano plot of metabolites in DDGS of step 2 and step 3; (**F**) Volcano plot of metabolites in DDGS of step 1 and step 3.

As shown in Figure 5C–F, the volcano plot was used to compare the metabolites of different fermentation days to further screen the potential metabolic markers in the fermentation process. The metabolites of fermented DDGS were compared with the unfermented DDGS. There were 70 key differential metabolites. Among them, the number of the up-regulated compounds during fermentation was 59, while there were 11 down-regulated compounds. Comparing the metabolites of step 1 and step 2, 111 key different metabolites were obtained, of which 34 were up-regulated compounds and 77 were down-regulated compounds. Tyr-Tyr, 5′-Methylthioadenosine, Phenylacetaldehyde, etc. were continuously enriched during this fermentation stage, while the contents of Sucrose, Ethyl glycolate, PyroGlu-Glu-Arg, 2,3-Dihydroxy-3-methylbutyric acid, Phe-Val-Arg decreased. These compounds might be the key influencing metabolites for the transition from the first stage to the second stage of fermentation. When the second stage of fermentation was transformed to the third stage, a total of 117 key changed compounds were screened out. During this process, 77 compounds were up-regulated, such as Hydroxyproline, Val-His, Ile-Ile-Arg, Met-Leu-Arg, N-Acetyl-L-aspartic acid, Asp-Asp, etc. while 40 compounds were down-regulated, such as (−)-Quinic acid, 1,4-D-Xylobiose, etc. When comparing the initial fermentation stage with the final fermentation stage, 118 different metabolites were screened out. These compounds were not the union of the above metabolites screened. It indicated that the metabolites of this fermentation process were dynamic. However, there were also some compounds that were continuously accumulated or continuously decreased during the whole fermentation process.

To achieve a comprehensive understanding of metabolite changes during the fermentation of DDGS by *A. niger*, based on the results of the compounds screened above, the pathway of each fermentation stage was analyzed via MetaboAnalyst 5.0. As shown in Figure 5B, 24 regulatory pathways were found, which mainly focused on the synthesis and metabolism of amino acids and sugars. The pathways of starch and sucrose metabolism, galactose metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway, and fructose and mannose metabolism were active in *A. niger* fermented DDGS. This result was consistent with the changes in the sugars mentioned above. In terms of amino acids, valine, leucine and isoleucine degradation; glycine, serine and threonine metabolism; tryptophan metabolism; valine, leucine and isoleucine biosynthesis; taurine and hypotaurine metabolism; β-alanine metabolism; cysteine and methionine metabolism; phenylalanine metabolism; and arginine and proline metabolism were found to be active. These active pathways also matched the amino acid changes described above.

The starch and sucrose metabolism mainly started from cellulose during DDGS fermented with *A. niger*. Butyrate was one of the degradation products of cellulose [37]. Butanoate metabolism might affect the degradation efficiency of cellulose. Arginine and proline metabolism; taurine and hypotaurine metabolism; alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism; and pantothenate and CoA biosynthesis were also related to the metabolic process of cellulose derivatives. They might also be the main pathways that affected cellulose degradation during fermentation. The degradation of cellulose in DDGS by *A. niger* could be controlled through these pathways. Besides, lysine and arginine biosynthesis were very important for the application of fermented DDGS in swine diets. Through the results of metabolic pathway screening, it could also be found that taurine and hypotaurine metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; and phenylalanine metabolism were correlated with lysine and arginine biosynthesis. Further, pantothenate and CoA biosynthesis; arginine and proline metabolism; and purine metabolism may indirectly affect lysine and arginine biosynthesis and cellulose degradation during DDGS fermentation with *A. niger* via affecting taurine and hypotaurine metabolism; alanine, aspartate and glutamate metabolism; or glycine, serine and threonine metabolism. It indicated that the amino acid biosynthesis and cellulose degradation that occurred in *A. niger* were interactional processes during fermenting DDGS. DDGS fermented with *A. niger* may mainly degrade cellulose to produce amino acids.

3.5. Key regulatory Pathways Identified and Mapped

Based on the above, the content changes of related compounds in key regulatory pathways during DDGS fermentation by *A. niger* were detected and analyzed. The signaling networks associated with differentially expressed metabolic pathways were mapped as well. According to the key metabolites identified and quantified, a metabolic interaction network including cellulose degradation, the tricarboxylic (TCA) cycle, threonine biosynthesis, lysine biosynthesis, arginine biosynthesis, etc. was made (Figure 6). During fermentation, *A. niger* would convert the cellulose in DDGS into glucose. Part of glucose was accumulated as a metabolite, while part was further converted into pyruvate to participate in the TCA cycle. 3-Phosphoglyceric acid and Phosphoenolpyruvate were one of the most important intermediates for producing amino acids [38]. Serine (Ser), Gly, and Thr came from 3-Phosphoglyceric acid, while Phe and Tyr came from Phosphoenolpyruvate. The synthesis of Lys, Arg, and Asp was associated with intermediates of the TCA cycle. α-Ketoglutarate (AKG) was an important precursor of Lys and Arg during *A. niger* fermented DDGS. Oxaloacetate was an important prerequisite for Asp. During fermentation, Pro could convert to Hydroxyproline and eventually AKG, providing more precursors for Lys and Arg synthesis. Metabolites accumulated and transformed in the whole DDGS fermentation process via *A. niger* interacted with each other. Finally, the metabolic pathways during DDGS fermentation with *A. niger* all pointed to an increase in Lys, Arg and Thr and a decrease in cellulose and Leu. This was consistent with the results of nutrient composition changes before and after fermentation.

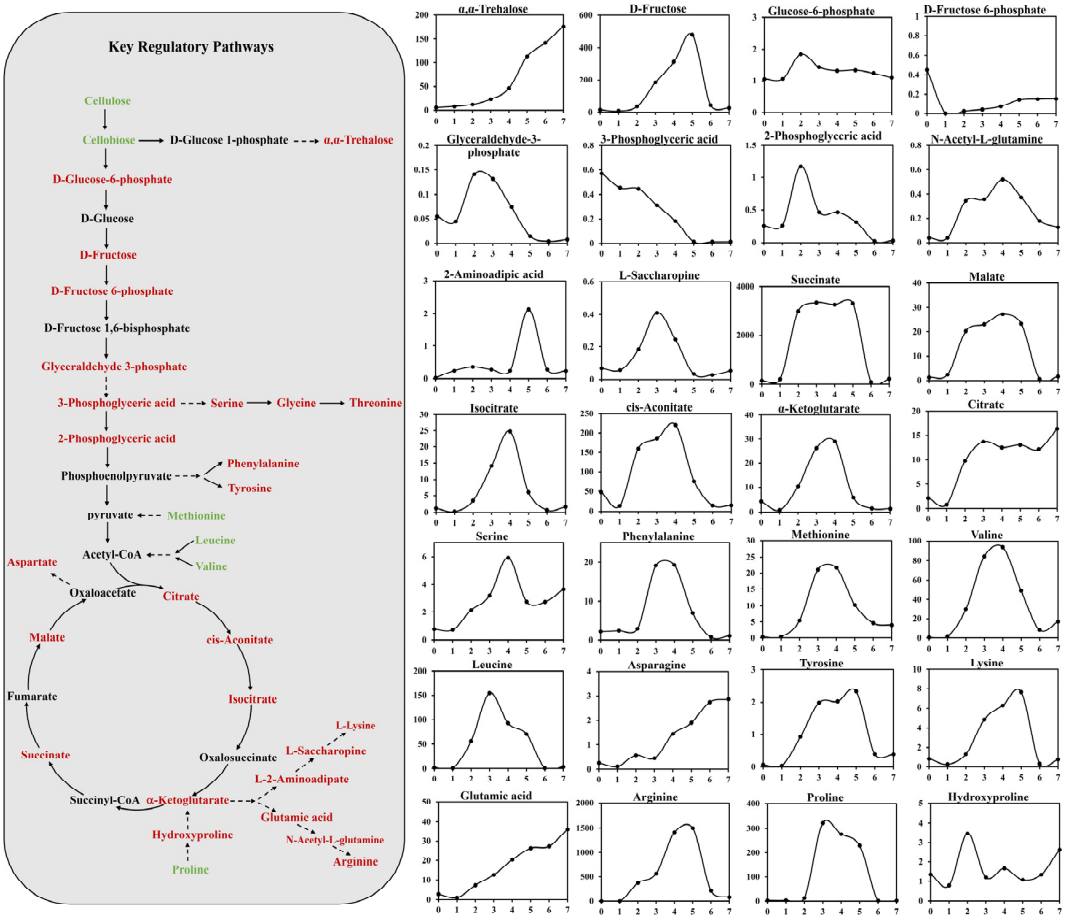

Figure 6. Key regulatory pathways and the key compounds concentration changes in the process of DDGS fermented by *Aspergillus niger.* Proposed metabolic pathways were based on the literature and KEGG database of metabolic pathways. The concentration unit was μg/g. The fermentation time was 1–7 days. Note: KEGG: Kyoto Encyclopedia of Genes and Genomes.

4. Conclusions

This study indicated that the feeding value of DDGS for pigs could be improved via *A. niger* fermentation and its key regulatory pathways. *A. niger* could degrade cellulose in DDGS to produce amino acids. In the early stage of fermentation, *A. niger* mainly degraded cellulose and synthesized Lys and Arg, while the synthesis of Asp occurred in the last stage. AKG was the key factor to regulate *A. niger* degrading cellulose and producing Lys and Arg. Pro could convert to Hydroxyproline and eventually AKG, facilitating Lys and Arg generation. Consequently, it proposed a nutrition-oriented remodeling of DDGS via *A. niger* under starch-carbon deficient conditions.

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