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Effect of *Aspergillus niger* Fermentation on the Metabolites in Corn Stalks

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Abstract: Fermentation has been considered as an effective means to improve the feed nutrient digestibility of corn stalks, and it is beneficial to animal growth performance and health. The beneficial functions of fermented corn stalks are related to the variety of metabolites produced through fermentation, but the nature of these components is still unclear. In this study, gas chromatography–mass spectrometry, combined with principal component analysis and partial least squares discriminant analysis, was used to explore the differential metabolites of corn stalks before and after *Aspergillus niger* fermentation. A total of 32 potential characteristic compounds were obtained, mainly including sugar and glycoside derivatives, organic acids and their derivatives, alcohol compounds, benzene and its substituted derivatives, amino acids, phenolic compounds, and flavonoids. Compared with the metabolites in corn straw before fermentation, the relative content of D-threitol, mannitol-1-phosphate, coniferin, citrazinic, oxoglutaric acid, chenodeoxycholic acid, naproxen, 5-aminovaleric acid, vanillin, catechin, and UDP-glucuronic acid was significantly increased, and the relative content of *N*-acetylgalactosamine, heneicosanoic acid, chlorogenic acid, and adenosine was significantly decreased. Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that 20 metabolic pathways corresponded to the differential characteristic metabolites. The results of this study will provide theoretical support for the quality evaluation of fermented corn stalks and high-value product development in the future.

Keywords: *Aspergillus niger*; fermentation; metabolomics; corn straw; metabolites

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1. Introduction

Corn stalks are an important agricultural waste and are partially used as a source of dietary energy for ruminants. However, they have poor nutritional value, with low protein and high fiber content, which prevents the access of ruminal hydrolytic enzymes to cellulose and hemicellulose [1]. Ensiling, as an anaerobic digestion technology, is used to improve the forage quality of corn stalks for high-production ruminants [2]. Recent studies found that fungal fermentation has higher efficiency than ensiling in improving the feed nutrient digestibility and animal growth performance, milk yield, and meat quality [3,4]. Among the strains used, *Aspergillus niger* is widely distributed in nature and possesses an excellent ability to secrete proteins, and has been widely used in traditional food brewing and enzyme production fields [5].

During the fermentation process, *Aspergillus* produces various enzymes, including protease, cellulase, hemicellulase, and pectinase [6], which can break the physical barrier between hemicellulose and lignin, thereby improving the fiber digestibility and nutrient efficiency [7]. Moreover, *Aspergillus* fermentation products can stabilize the pH of the internal environment of the animal rumen [8], with beneficial effects on milk performance

in dairy cows [9]. Obviously, these benefits are not only caused by the degradation of cellulose and hemicellulose, but are also related to the beneficial substances accumulated during fermentation. These components may include a series of enzymolysis products and a variety of metabolites obtained through their own fermentation metabolism. For example, *A. niger* fermentation may promote the release of phenolic acids from hemicellulose and enhance the antioxidant properties of the fermented product [10], and it significantly increases the content of aromatic carbon and carboxyl [11]. Unfortunately, what exactly these components are is still unclear. Fortunately, with the help of emerging metabolomics technology [12,13], it is expected that we can determine the key metabolites of *Aspergillus*-fermented products and clarify their formation mechanism.

In this study, GC–MS technology was used to analyze the metabolome of *A. niger*-fermented corn stalks, and the differential metabolites were screened by multivariate statistical analysis and variable importance in projection value analysis. Then, the possible effects of differential metabolites on animal growth performance and immune responses were preliminary discussed. The study will provide theoretical support for the quality evaluation of *A. niger*-fermented corn stalks and high-value product development in the future.

2. Materials and Methods

2.1. Materials and Reagents

Corn stalks were supplied by Xinjiang Tecon Feed Technology Co., Ltd. Chloroform was purchased from Wokai Biotechnology Co., Ltd. (Shanghai, China). Methanol and acetonitrile were obtained from Fisher Chemical (Hampton, NH, USA) Pyridine was provided by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Methoxyamine hydrochloride and L-2-chlorophenylalanine were obtained from Adamas Technology Co., Ltd. (Shanghai, China). N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosilane, TMCS) was purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). Ten fatty acid methyl esters, including methyl caprylate, methyl nonaoate, methyl decanoate, methyl dodecanoate, methyl tetradecanoate, methyl hexadecanoate, methyl octadecanoate, methyl arachidate, methyl docosanoate, and methyl tetracosanoate, were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA).

2.2. Methods

2.2.1. Solid-State Fermentation

The laboratory-preserved *A. niger* HQ2 was selected as the fermentation strain, and it was activated in PDA (30 °C, 5 d). Tween 80 solution (0.01%) was used to recover the spores. The spores were calculated by counting with Neubauer's chamber, and were adjusted to 10^8 spores/mL for further use. Corn stalks in a volume of 95 g were supplemented with 2.5 g ammonium sulfate, 1.5 g dipotassium hydrogen phosphate, 0.6 g magnesium sulfate, 0.2 g sodium chloride, 0.2 g calcium chloride, and 100 g water. Except for sodium chloride and calcium chloride dissolved in deionized water, the other substances were directly evenly mixed with corn stalks. Then, 10 mL of *A. niger* spore suspension (1×10^8 CFU/mL) was inoculated and fermented at 30 °C for 6 d. After fermentation, the sample was placed in a 60 °C oven to dry to a moisture content of about 12%, and pulverize to a 40-mesh pass rate of over 80% for use as a fermentation sample (Fer). At the same time, a sample with no inoculated strains was used as the control (Con).

2.2.2. Biochemical Analysis

Neutral detergent fiber (NDF) was determined by the national standard of the People's Republic of China (GB/T 20806-2006), and acid detergent fiber (ADF) was determined by the agricultural industry standard of the People's Republic of China (NY/T 1459-2022). Hemicellulose, cellulose, and lignin of corn stalk before or after fermentation were estimated using the method proposed by Waksman [14]. For the hemicellulose determination, the sample is extracted with cold-water, hot-water, and alcohol step by step, and the residue is treated by 2% dilute hydrochloric acid. The sugar in the extract is determined, and the

hemicellulose content is calculated. In case of cellulose, the residue above is treated with 10 volumes of sulfuric acid, and then autoclaved at 121 °C for 1 h after 15 volumes of water was added. Following that, it is neutralized with 10% sodium hydroxide solution and filtering, and the sugar is determined. The residue from the above sulfuric acid treatment is thoroughly washed, dried, and weighed. The lignin content was calculated by subtracting the ash and crude protein content according to ordinary methods.

2.2.3. Sample Preparation for GC–MS

The fermentation and control samples were freeze-dried with a vacuum freeze dryer (Scientz-100F). The freeze-dried samples were pulverized using a mixer with zirconia beads (MM 400, Retsch) at 30 Hz for 1.5 min. We dissolved 100 mg of lyophilized powder in 1.2 mL of 70% methanol solution, vortexed it for 30 s every 30 min 6 times, and placed the samples in a 4 °C refrigerator overnight. After centrifugation at 12,000× *g* for 10 min, the extract was filtered (SCAA-104) using a filter with a 0.22-micron pore size and the filtrate was evaporated to dryness. Methoxyamine hydrochloride pyridine solution (15 mg/mL, 80 µL) was added and then oximation reaction was performed at 37 °C for 90 min. A volume of 50 µL BSTFA (containing 1% TMCS) derivatization reagent and 20 µL n-hexane were added into the samples, and then the internal standards (ten fatty acid methyl esters and L-2-chlorophenylalanine) were added subsequently to react at 70 °C for 60 min.

2.2.4. GC–MS Analysis

The derivatized samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977B MSD system (Agilent Technologies Inc., Santa Clara, CA, USA) in the split mode for analysis, with an injection volume of 1 µL and a split ratio of 10:1. The samples were separated by a DB-5MS capillary column (40 m × 0.25 mm, 0.25 µm) and then subjected to mass spectrometry detection. The injection port temperature was 260 °C, the carrier gas was high-purity helium, the carrier gas flow rate was 1 mL/min, the septum purge flow rate was 3 mL/min, and the solvent delay was 5 min. Heating program: the initial temperature was 60 °C, equilibrated for 0.5 min, then increased to 310 °C at 8 °C/min, and maintained for 6 min. Electron ionization source; transmission line temperature 310 °C; ion source temperature 230 °C; quadrupole temperature 150 °C; electron energy 70 eV. The scanning mode was full, the mass scanning range was m/z 50–500, and the scanning frequency was 3.2 scan/s.

2.2.5. Data Processing, Statistical Analysis, and Identification of Metabolites

All GC–MS spectra were processed in MassHunter Workstation Quantitative Analysis (v10.0.707.0) software to perform peak extraction, alignment, and other data preprocessing operations, and we input the obtained data into ropls (Version1.6.2) software, using R2 and Q2, for principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). According to a p value on Student's t-test of less than 0.05 and the calculated variable importance in the projection (VIP) value of PLS-DA model PC > 1, differential metabolites were screened.

Differential metabolites among two groups were summarized and mapped onto their biochemical pathways through metabolic enrichment and pathway analysis based on a database search (Kyoto Encyclopedia of Genes and Genomes, KEGG, http://www.genome.jp/kegg/, accessed on 18 May 2022). These metabolites can be classified according to the pathways they are involved in or the functions they performed. Pathways with significantly changed and mapped metabolites were then fed into metabolite sets enrichment analysis. Enrichment analysis of KEGG pathways was performed using Fisher's exact test. Statistical analyses were performed using R software v3.6.2.

The samples were analyzed six times and the results are presented as the means ± standard errors. The differences among the Fer and Con samples were analyzed using a t-test. Two-sided values of $p < 0.05$ or $p < 0.01$ were considered statistically significant.

3. Results and Discussion

3.1. Biochemical Changes in Fermented Corn Stalks

The effects of *A. niger* fermentation on the composition of the corn stalks are shown in Table 1. There were differences among fermented and control samples in the Neutral detergent fiber (NDF) ($p < 0.01$), hemicellulose ($p < 0.01$), and crude protein ($p < 0.01$). *A. niger* fermentation reduced the NDF content of corn stalks from 66.98% to 58.46%. Degradation of lignocellulose content in the corn stalks was caused by the reduction in the hemicellulose and cellulose content of corn stalks, which was attributed to the activity of cellulase, β-glucosidase, and xylanase in *A. niger* [15]. In addition, the crude protein content of the fermented corn stalks significantly ($p < 0.01$) increased from 8.20% to 11.26%. Although the nutritional value of corn stalks was modified by *A. niger* fermentation, we think these biochemical changes cannot fully explain the beneficial effects on animal growth performance and health.

Table 1. Effects of *A. niger* fermentation on the composition of corn stalks (% Dry matter).

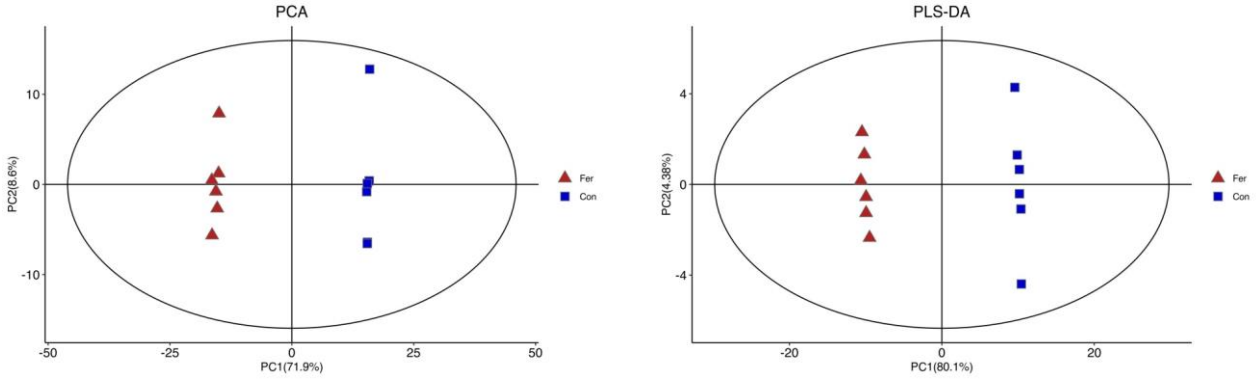
Fractions	Control	Fermented
Neutral detergent fiber	66.98 ± 0.08	58.46 ± 0.28
Acid detergent fiber	38.12 ± 0.48	36.96 ± 0.64
Lignin	5.24 ± 0.26	5.14 ± 0.32
Cellulose	33.92 ± 0.42	31.36 ± 0.62
Hemicellulose	23.86 ± 0.54	20.06 ± 0.22
Crude protein	8.20 ± 0.16	11.24 ± 0.18

3.2. Multivariate Statisitical Analysis of Detected Metabolites

To visualize the variability and overall distribution trends among sample groups and within groups, principal component analysis (PCA) was performed for the metabolites detected in fermentation samples (Fer) and control samples (Con). The first and second principal components accounted for 71.9% and 8.6% of the total variance, respectively (Figure 1A). In the PCA score plot, the Fer and Con samples were concentrated in this group and were clearly separated between the groups, indicating that there was a clear grouping trend between the two groups of samples, which had analytical value. Partial least squares discriminant analysis (PLS-DA) is a multivariate statistical analysis method for supervised pattern recognition (Figure 1B). The specific method extracts the components of the independent variable X and the dependent variable Y and then calculates the correlation between the components. Compared with PCA, PLS-DA can maximize the distinction between groups, which is beneficial in identifying differential metabolites. To differentiate among the major metabolites between Fer and Con samples, the PLS-DA method was used to maximize sample separation. The PLS-DA score plot showed that the Fer and Con samples are clearly separated, and the model had a strong ability to explain sample differences.

3.3. Differential Metabolite Analysis Results and Visualization

To identify the key differential metabolites in the Fer and Con samples, the variable importance in the projected (VIP) values of the first principal component of the PLS-DA model, and the *p* value of the *t*-test were used to identify the differential metabolism in the two groups of samples, and 370 differential metabolites were identified. Among them, the total number of increased metabolites was 172 (Fold Change, FC > 1), the total number of decreased metabolites was 128 (FC < 1), and the total number of unchanged metabolites was 70. Metabolite differences between Fer and Con samples were visualized by volcano plots, as shown in Figure 2.



(**A**) (**B**)

Figure 1. PCA (**A**) and PLS-DA (**B**) score plots of the samples before and after *A. niger* fermentation (n = 6). Note: red triangle (fermentation sample) and blue square (control sample).

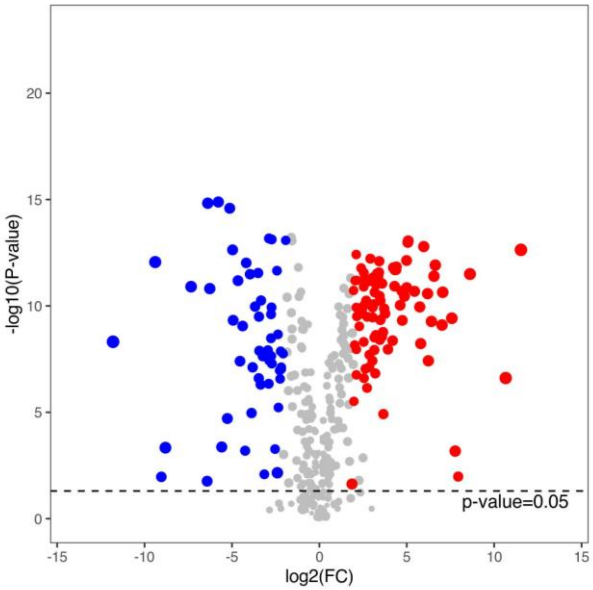


Figure 2. Volcano plot for the differential metabolites of the samples before and after *A. niger* fermentation (n = 6). The red circle indicates an increase, the blue circle denotes a decrease, and the gray circle indicates a lack of significance.

To better show the metabolite differences between the two groups of samples, a metabolite heatmap for a total of 12 samples in two groups was drawn (Figure 3). From the obtained heatmap, it could be seen that the concentrations of metabolites in the two groups of samples were very different; some metabolites were significantly higher in the Fer group than in the Con group, and some metabolites in the Con group were significantly higher than in the Fer group, so it was necessary to further screen these metabolites with large differences in content.

A total of 32 significantly different metabolites were identified from the two sets of samples with VIP $\geq$ 1.5, FC $\geq$ 2.0 or FC $\leq$ 0.5, and $p \leq 0.05$. Among them, 20 metabolites were increased and 12 were decreased. Differential characteristic metabolites mainly include sugars and their derivatives, organic acids, alcohol compounds, benzene, substituted derivatives, amino acids, phenolic compounds, flavonoids, and other compounds. The specific information on differential characteristic metabolites is shown in Table 2.

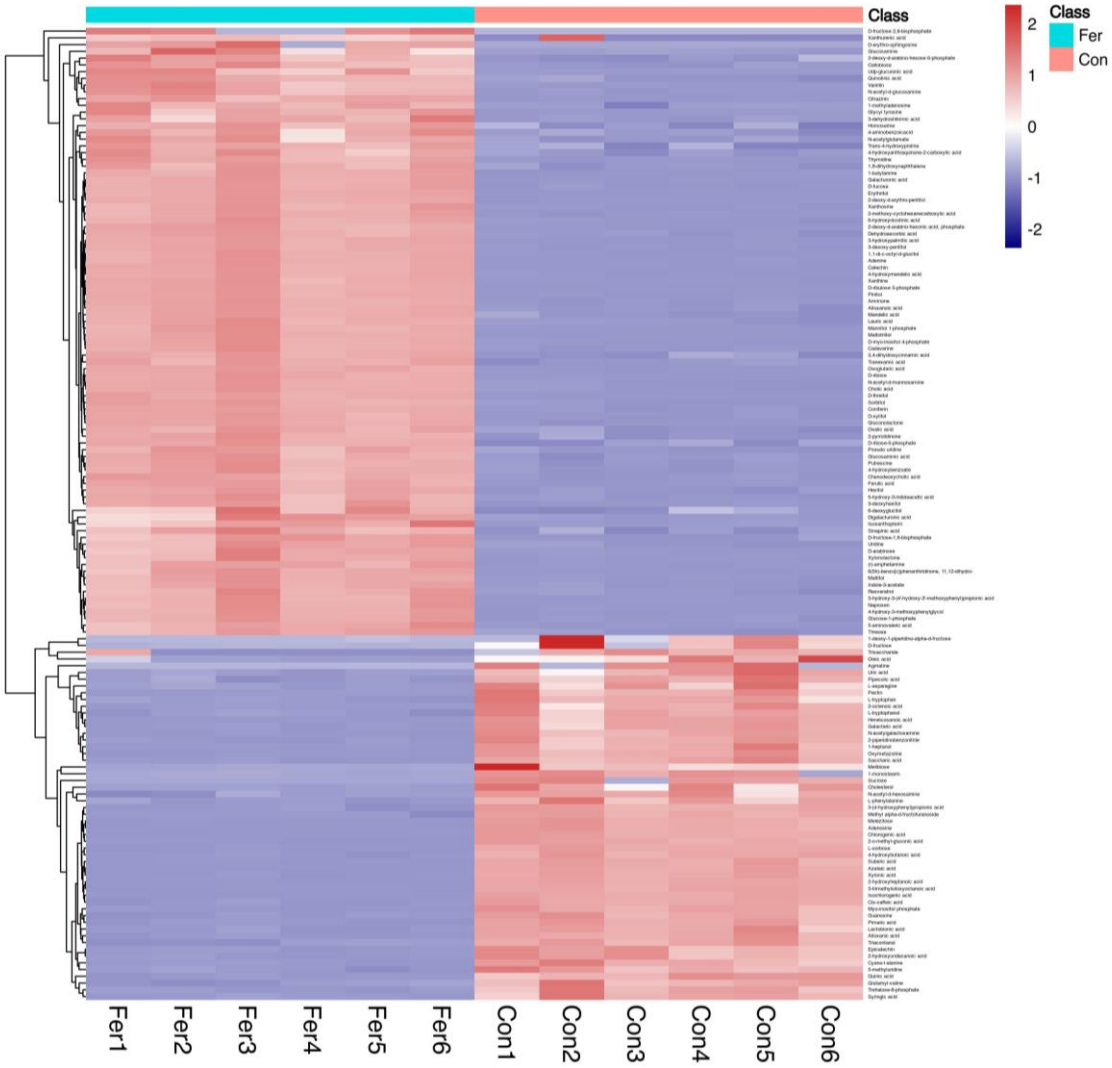


Figure 3. Heatmap of hierarchical clustering analysis for the differential metabolites of the samples before (Con) and after (Fer) *A. niger* fermentation (n = 6).

3.3.1. Sugar and Glycoside Derivatives

As the main carbon source, carbohydrates are consumed in *Aspergillus* fermentation and provide the necessary energy for the growth of *A. niger* through carbohydrate metabolism [16]. The 10 types of sugar and glycoside derivatives identified in the metabolite differences may be related to the improvement in the nutritional value of the fermented corn stalks. They were D-threitol, D-ribulose-5-phosphate, mannitol-1-phosphate, coniferin, erythritol, *N*-acetyl-D-mannosamine, *N*-acetylgalactosamine, melibiose, melezitose, and sucrose. Among them, the content of D-threitol increased after *A. niger* fermentation, and this substance has a non-specific protective effect on the organism and can be used as an osmotic protective agent and an antifreeze agent at extremely low temperatures [17]. It can also be used as an anticancer drug [18] and as a synthetic phospholipid [19]. Ribulose-5-phosphate is a carbonic acid receptor in photosynthesis. It is produced by ribulose phosphate kinase in the pentose phosphate cycle and is phosphorylated by ATP to produce ribulose-1, 5-diphosphate. Mannitol-1-phosphate is an important polyol, and its natural products are widely found in plants, algae, and other organisms. Studies have shown that the addition of sugar alcohols during microbial fermentation can promote the synthesis of secondary metabolites [20]. The content of coniferin, as an efficient antioxidant, was significantly increased, mainly due to the degradation of lignin [21]. Erythritol is synthesized in fungi through the pentose phosphate pathway [22]. It can replace sugar and be used as a food

additive and antioxidant [23]. In addition, it has a positive effect on intestinal flora [24]. The decrease of *N*-acetylgalactosamine indicated that the *Aspergillus*-fermented product had better safety. Similar to liver pathological changes in clinical viral hepatitis, this substance is a hepatocyte uridine phosphate interfering agent, which can cause diffuse necrosis and inflammation of the liver [25]. The decline in melibiose, melezitose, and sucrose was mainly due to their consumption in the process of microbial growth and metabolism.

Table 2. Thirty-two differential characteristic metabolites associated with *Aspergillus* fermentation.

Species	Metabolite Name	VIP	log_2(FC)	Increase/Decrease
Sugar and glycoside derivatives	D-Threitol	2.45	11.53	Increase
	D-Ribulose-5-phosphate	2.13	8.62	Increase
	Mannitol-1-phosphate	1.73	5.74	Increase
	Coniferin	1.64	5.08	Increase
	Erythritol	1.61	4.99	Increase
	N-Acetyl-D-mannosamine	1.52	4.41	Increase
	N-Acetylgalactosamine	1.52	−4.41	Decrease
	Melibiose	1.70	−5.59	Decrease
	Melezitose	1.96	−7.34	Decrease
	Sucrose	2.06	−8.81	Decrease
Organic acids and derivatives	Citrazinic	1.92	7.06	Increase
	3-Hydroxypalmitic acid	1.77	5.97	Increase
	Oxoglutaric acid	1.63	5.06	Increase
	Chenodeoxycholic acid	1.60	4.87	Increase
	Cholic acid	1.52	4.39	Increase
	Xanthurenic acid	1.72	1.87	Increase
	Azelaic acid	1.61	−4.96	Decrease
	Heneicosanoic acid	2.48	−11.8	Decrease
Alcohol compounds	D-myo-Inositol-4-phosphate	1.57	4.72	Increase
	1-Heptanol	1.54	−4.54	Decrease
	Cholesterol	1.65	−5.26	Decrease
	Isochlorogenic acid	1.82	−6.38	Decrease
	Chlorogenic acid	2.21	−9.39	Decrease
Benzene and substituted derivatives	(r)-Amphetamine	1.55	4.62	Increase
	Oxymetazoline	1.60	−4.93	Decrease
	Naproxen	1.83	6.41	Increase
Amino acids	5-Aminovaleric acid	1.57	4.74	Increase
Phenolic compounds	Vanillin	1.81	6.24	Increase
Flavonoids	Catechin	1.87	6.63	Increase
Other compounds	UDP-Glucuronic acid	2.36	10.66	Increase
	D-erythro-Sphingosine	1.85	7.77	Increase
	Adenosine	1.81	−6.27	Decrease

3.3.2. Organic Acids and Their Derivatives

Organic acids has been reported for immune potentiating, antibacterial, and growth promoters in livestock and poultry [26,27]. We accurately identified eight organic acids and their derivatives, including citrazinic, 3-hydroxypalmitic acid, oxoglutaric acid, chenodeoxycholic acid, cholic acid, xanthurenic acid, azelaic acid, and heneicosanoic acid. The increase in the metabolic content of citrazinic and oxoglutaric acid mainly reflects their roles as antibacterial and anti-inflammatory agents [28,29]. As trace components in the feed, they have excellent reactivity and good value for the growth and development of poultry. The increased content of chenodeoxycholic acid and cholic acid has anti-inflammatory and antibacterial medicinal value for poultry feeding, and they can dissolve cholesterol stones [30]. Chenodeoxycholic acid can improve the intestinal absorption of vitamins and lipids and also improve the intestinal damage caused by endotoxins [31]. Cholic acid has a

certain therapeutic effect on malignant liver tumors and it could increase the pain threshold and lower the body temperature in the mice [32]. Heneicosanoic acid is a free fatty acid, it can be converted into neutral lipids and stored in cytosolic lipid droplets. When the energy required is insufficient, it will be decomposed into free fatty acids again for energy use [33].

3.3.3. Alcohol Compounds

Alcohols mainly provide a specific aroma or spicy taste in fermented products. Five alcohol metabolites with significant differences were identified and screened, namely D-myo-inositol-4-phosphate, 1-heptanol, cholesterol, isochlorogenic acid, and chlorogenic acid. Although the addition of cholesterol can improve the growth performance of poultry to a certain extent [34], it was also found that the content of cholesterol in various tissues in crayfish and the content of high-density lipid cholesterol in the hemolymph also increased with the increase in the level of dietary cholesterol, affecting the normal body fat metabolism and reducing the antioxidant capacity of the shrimp hemolymph. The decrease of isochlorogenic acid and chlorogenic acid is mainly due to the degradation of chlorogenic acid isomers to volatile components [35], or it may be linked to various enzymes produced by microbial fermentation.

3.3.4. Benzene and Its Substituted Derivatives

The benzene family causes great harm to the human body and poultry in daily production and life. The content of oxymetazoline, a differential metabolite identified in this work, is decreased, mostly due to the consumption of biosynthesis in the process of microbial metabolism; meanwhile, regarding naproxen, the acid content rises, and it mainly acts as a trace drug component to play the role of an anti-oxidation, antipyretic, and analgesic agent. It has good antipyretic and anti-inflammatory effects in mice [36].

3.3.5. Amino Acids

A significantly different amino acid was accurately identified, and its content was increased—namely, α-aminovaleric acid. Amino acids mainly arise from the enzymatic degradation of proteins in raw materials by proteases produced by microorganisms during the fermentation process of *Aspergillus* and the autolysis of other microorganisms. Alpha-aminovaleric acid, also known as norvaline, is mainly used as an antibacterial substance and an inhibitor of certain enzymes in feed. De Abhijit found that by injecting L-norvaline into male rats, the inhibition of arginase L-norvaline could increase the serum nitrate, urea, LDH, testosterone, and testicular protein levels, thereby improving diabetes-induced sexual dysfunction [37].

3.3.6. Phenolic Compounds

Phenolic substances mainly play an antioxidant role in feed, and vanillin was identified with significantly increased content in this work. It is a type of aroma substance formed by biotransformation, such as the non-oxidative decarboxylation reaction of ferulic acid to 4-vinyl guaiacol and then converted into vanillin during the fermentation process of *Aspergillus* [38]. It has a variety of physiological functions, such as anti-oxidation, anti-inflammatory [39], and antibacterial, and it causes the inhibition of some chronic diseases through self-polymerization and free radical scavenging [40].

3.3.7. Flavonoids

Flavonoids have a variety of bioactive functions, such as antibacterial, antioxidant, antiviral, and antitumor [41,42]. Plant flavonoids are a type of safe and green feed additive [43], and they can improve the growth performance, production performance, and the reproductive ability of livestock and poultry [44]. Catechin has good application value. In feed, it can act as an antioxidant [45], and is an antibacterial, anticancer, antihyperlipidemic, antidiabetic, and anti-inflammatory agent [46]. At the same time, studies have found that catechins can also improve lipopolysaccharide-induced intestinal inflammation

in piglets [45] and they can regulate certain corresponding signaling pathways to inhibit cellular inflammation [47] and acute pancreatitis disease symptoms [48].

3.3.8. Other Compounds

UDP-Glucuronic acid, D-erythro-sphingosine, and adenosine were also identified among the differential metabolites of *Aspergillus*-fermented corn stalks. The increase in the content of UDP-glucuronic acid is caused by the metabolism of *Aspergillus*. It is an important glycosyl donor in cells and an essential precursor for structural polysaccharides, hyaluronic acid, cell growth, and metabolism [25]. The content of D-erythro-sphingosine also increased to a certain extent, and it plays an important role in regulating diverse cellular processes, such as cell growth, differentiation, and apoptosis [49]. The content of adenosine decreased after fermentation, and the main degradation enzymes were adenosine deaminase and adenosine kinase. Intracellular adenosine is phosphorylated by adenosine kinase or converted to inosine by adenosine deaminase, resulting in reduced levels [50].

3.4. Metabolic Pahtway Analysis

The most important biochemical metabolic pathways and signaling pathways associated with the differential metabolites were identified. The differential metabolites identified were enriched by the KEGG pathway, and the obtained KEGG enrichment pathway (top 20) bubble chart is shown in Figure 4. The abscissa of the figure is the ratio of the number of differential metabolites identified in the corresponding pathway to the total number of metabolites. As can be seen from Figure 4, the ABC transporter pathway (ko02010), pentose and glucuronate interconversion (ko00040), the phosphotransferase system (ko02060), the central carbon metabolism pathway in cancer (ko05230), D-amino acid metabolism (ko00470), purine metabolism (ko00230), the protein digestion and absorption pathway (ko04974), and ascorbate and aldarate metabolism (ko00053) were associated with the significantly differential metabolites in *Aspergillus* fermentation, with highly differential metabolite enrichment and high reliability.

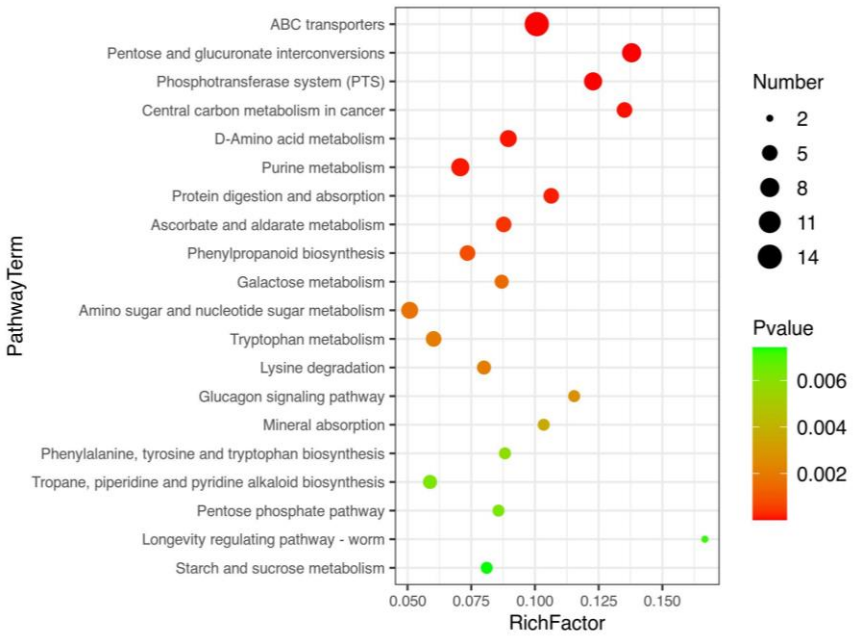


Figure 4. Enrichment analysis of pathways (top 20) associated with the differential metabolites.

Combined with the screened differential metabolites, it was found that adenosine, erythritol, and sucrose were directly bound with ABC transporters (ko02010), and this pathway can translocate a broad spectrum of molecules across the cell membrane; UDP-glucuronic acid and D-ribulose-5-phosphate were related to pentose and glucuronate

interconversion (ko00040), D-ribulose-5-phosphate and mannitol-1-phosphate were related to the phosphotransferase system (ko02060), and this metabolic pathway is involved in both the transport and phosphorylation of a large number of carbohydrates and in the regulation of a number of other metabolic pathways; adenosine, UDP-glucuronic acid, and *N*-acetyl-d-mannosamine were related to D-amino acid metabolism (ko00470); oxoglutaric acid was related to ascorbate and aldarate metabolism (ko00053). Associated with increased levels of 5-aminovaleric acid were starch and sucrose metabolism (ko00520), tryptophan metabolism (ko00380), lysine degradation (ko00310), and phenylalanine, tyrosine, and tryptophan biosynthesis (ko00400).

4. Conclusions

Here, we used GC–MS technology to analyze the metabolites of corn stalks before and after *A. niger* fermentation. The results showed that *A. niger* fermentation had a significant effect on the metabolites in corn stalks. Through the PCA and PLS-DA results, it was found that the metabolites in the fermentation group and the control group were significantly separated, indicating that these metabolites were significantly different. Based on VIP $\geq$ 1.5, FC $\leq$ 0.5 or FC $\geq$ 2, and $p \leq 0.05$, a total of 32 differential characteristic metabolites were screened, mainly including sugar and glycoside derivatives, organic acids and their derivatives, alcohol compounds, amino acids, and flavonoids. Compared with the control group, the content of D-threitol, mannitol-1-phosphate, coniferin, citrazinic, oxoglutaric acid, chenodeoxycholic acid, naproxen, 5-aminovaleric acid, vanillin, catechin, and UDP-glucuronic acid was significantly increased, whereas the content of *N*-acetylgalactosamine, heneicosanoic acid, chlorogenic acid, and adenosine was significantly decreased. KEGG pathway analysis showed that 20 metabolic pathways corresponded to the differential characteristic metabolites. The study will provide theoretical support for the quality evaluation of fermented corn stalks and high-value product development in the future.

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References

1. Gado, H.M.; Elghandour, M.M.Y.; Cipriano, M.; Odongo, N.E.; Salem, A.Z.M. Rumen degradation and nutritive utilization of wheat straw, corn stalks and sugarcane bagasse ensiled with multienzymes. *J. Appl. Anim. Res.* **2017**, *45*, 485–489. [CrossRef]
2. Wang, Y.; Xia, K.; Wang, X.N.; Lin, X.; Liu, J.; Li, Y.J.; Liu, X.L.; Zhao, W.J.; Zhang, Y.G.; Guo, J.H. Improvement of feed intake, digestibility, plasma metabolites, and lactation performance of dairy cows fed mixed silage of sugar beet pulp and rice straw inoculated with lactic acid bacteria. *J. Dairy Sci.* **2022**, *105*, 269–280. [CrossRef] [PubMed]
3. Xiang, H.; Zhao, X.L.; Fang, Y.; Wang, F.; Liang, R.; Sun, X.Z.; Wang, S.P.; Zhong, R.Z. Feeding fungal-pretreated corn straw improves health and meat quality of lambs infected with gastrointestinal nematodes. *Animals* **2020**, *10*, 1659. [CrossRef]

4. Zhao, X.L.; Wang, F.; Fang, Y.; Zhou, D.W.; Wang, S.P.; Wu, D.Q.; Wang, L.X.; Zhong, R.Z. High-potency white-rot fungal strains and duration of fermentation to optimize corn straw as ruminant feed. *Bioresour. Technol.* **2020**, *312*, 123512. [CrossRef]
5. Liu, Y.; Yue, Z.; Jinglei, L.; Shan, L.; Shudong, H.; Hanju, S.; Shengfei, Y.; Shangying, X. Effect of enzymes addition on the fermentation of Chinese rice wine using defined fungal starter. *LWT-Food Sci. Technol.* **2021**, *143*, 111101.
6. Jeong, E.; Seo, J.-A. Enzyme activity of *Aspergillus* section Nigri strains isolated from the Korean fermentation starter, nuruk. *J. Microbiol.* **2022**, *60*, 998–1006. [CrossRef] [PubMed]
7. Di Francia, A.; Masucci, F.; De Rosa, G.; Vafficchio, M.L.; Proto, V. Effects of *Aspergillus oryzae* extract and a *Saccharomyces cerevisiae* fermentation product on intake, body weight gain and digestibility in buffalo calves. *Anim. Feed. Sci. Technol.* **2008**, *140*, 67–77. [CrossRef]
8. Nedelkov, K.; Raisanen, S.E.; Chen, X.; Harper, M.T.; Melgar, A.; Oh, J.; Compart, D.M.P.; Hristov, A.N. Effect of enzyme extracts from *Aspergillus oryzae* and *Aspergillus niger* on rumen bacterial and fungal diversity and fermentation in vitro. *J. Dairy Sci.* **2019**, *102*, 378–379.
9. Zhang, J.Y.; Jin, W.; Jiang, Y.; Xie, F.; Mao, S.Y. Response of milk performance, rumen and hindgut microbiome to dietary supplementation with *Aspergillus oryzae* fermentation extracts in dairy cows. *Curr. Microbiol.* **2022**, *79*, 113. [CrossRef]
10. Yin, Z.N.; Wu, W.J.; Sun, C.Z.; Lei, Z.G.; Chen, H.M.; Liu, H.F.; Chen, W.B.; Ma, J.J.; Min, T.; Zhang, M.M.; et al. Comparison of releasing bound phenolic acids from wheat bran by fermentation of three *Aspergillus* species. *Int. J. Food Sci. Technol.* **2018**, *53*, 1120–1130. [CrossRef]
11. Yang, Y.A.; Wang, L.L.; Zhang, Y.F.; Li, L.B.; Shi, X.Y.; Liu, X.T.; Ren, X.D.; Dou, S. Transformation of corn stalk residue to humus-like substances during solid-state fermentation. *Sustainability* **2019**, *11*, 6771. [CrossRef]
12. Yang, H.; Xie, Y.; Li, X.M.; Wu, D.H.; Cai, G.L.; Lu, J. Key compounds and metabolic pathway responsible for the browning in Dangshan pear (*Pyrus* spp.) wine. *J. Agric. Food Chem.* **2021**, *69*, 10311–10320. [CrossRef]
13. Wang, Y.B.; Lin, B.J.; Li, Z.X. Effect of *Lactobacillus plantarum* fermentation on metabolites in lotus leaf based on ultra-high-performance liquid chromatography-high-resolution mass spectrometry. *Fermentation* **2022**, *8*, 599. [CrossRef]
14. Waksman, S.A.; Stevens, K.R. A system of proximate chemical analysis of plant materials. *Ind. Eng. Chem. Anal. Ed.* **1930**, *2*, 167–173. [CrossRef]
15. Daly, P.; van Munster, J.M.; Blythe, M.J.; Ibbett, R.; Kokolski, M.; Gaddipati, S.; Lindquist, E.; Singan, V.R.; Barry, K.W.; Lipzen, A.; et al. Expression of *Aspergillus niger* CAZymes is determined by compositional changes in wheat straw generated by hydrothermal or ionic liquid pretreatments. *Biotechnol. Biofuels Bioprod.* **2017**, *10*, 35. [CrossRef]
16. Lee, D.E.; Lee, S.; Jang, E.S.; Shin, H.W.; Moon, B.S.; Lee, C.H. Metabolomic profiles of *Aspergillus oryzae* and *Bacillus amyloliquefaciens* during rice koji fermentation. *Molecules* **2016**, *21*, 773. [CrossRef]
17. Berks, A.H. The chapman and hall dictionary of drugs on CD-ROM. *J. Chem. Inf. Comput. Sci.* **1995**, *35*, 332–333. [CrossRef]
18. Kopfmaier, P.; Sass, G. Antitumor-activity of treosulfan against human breast carcinomas. *Cancer Chemother. Pharmacol.* **1992**, *31*, 103–110. [CrossRef]
19. Sommerdijk, N.; Feiters, M.C.; Nolte, R.J.M.; Zwanenburg, B. Aggregation behavior of a phospholipid based on D-(-)-threitol. *J. R. Neth. Chem. Soc.* **1994**, *113*, 194–200.
20. Shen, Y.; Gu, L.; Zhang, J.; Chen, J.; Du, G. Effects of mixed carbon sources on glucose oxidase production by recombinant Pichia pastoris. *Chin. J. Biotechnol.* **2013**, *29*, 927–936.
21. Hofmann, T.; Talos-Nebehaj, E.; Albert, L.; Nemeth, L. Antioxidant efficiency of Beech (*Fagus sylvatica* L.) bark polyphenols assessed by chemometric methods. *Ind. Crops Prod.* **2017**, *108*, 26–35. [CrossRef]
22. Janek, T.; Dobrowolski, A.; Biegalska, A.; Mironczuk, A.M. Characterization of erythrose reductase from *Yarrowia lipolytica* and its influence on erythritol synthesis. *Microb. Cell Factories* **2017**, *16*, 118. [CrossRef] [PubMed]
23. Flint, N.; Hamburg, N.M.; Holbrook, M.; Dorsey, P.G.; LeLeiko, R.M.; Berger, A.; de Cock, P.; Bosscher, D.; Vita, J.A. Effects of erythritol on endothelial function in patients with type 2 diabetes mellitus: A pilot study. *Acta Diabetol.* **2014**, *51*, 513–516. [CrossRef] [PubMed]
24. Mahalak, K.K.; Firrman, J.; Tomasula, P.M.; Nunez, A.; Lee, J.J.; Bittinger, K.; Rinaldi, W.; Liu, L.S. Impact of steviol glycosides and erythritol on the human and cebus apella gut microbiome. *J. Agric. Food Chem.* **2020**, *68*, 13093–13101. [CrossRef] [PubMed]
25. Bapu, D.; Runions, J.; Kadhim, M.; Brooks, S.A. N-acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: A role for truncated O-glycans in metastatic mechanisms. *Cancer Lett.* **2016**, *375*, 367–374. [CrossRef]
26. Pearlin, B.V.; Muthuvel, S.; Govidasamy, P.; Villavan, M.; Alagawany, M.; Ragab Farag, M.; Dhama, K.; Gopi, M. Role of acidifiers in livestock nutrition and health: A review. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 558–569. [CrossRef]
27. Khan, R.U.; Naz, S.; Raziq, F.; Qudratullah, Q.; Khan, N.A.; Laudadio, V.; Tufarelli, V.; Ragni, M. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. *Environ. Sci. Pollut. Res.* **2022**, *29*, 32594–32604. [CrossRef]
28. Tomaszewska, E.; Dobrowolski, P.; Puzio, I. Postnatal administration of 2-oxoglutaric acid improves the intestinal barrier affected by the prenatal action of dexamethasone in pigs. *Nutrition* **2012**, *28*, 190–196. [CrossRef]
29. Sun, Y.Y.; Wang, C.H. Inhibitory effect of growth-inhibitor on Isochrysis galbana and inhibition of antioxidants on growth-inhibitor damage to its cells. *Prog. Biochem. Biophys.* **2007**, *34*, 1162–1168.
30. Hu, X.; Wang, J. Preparation and application of chenodeoxycholic acid and its derivatives. *Prog. Chem.* **2016**, *28*, 814–828.
31. Su, J.H.; Wang, H.X.; Ma, C.Y.; Liu, C.X.; Rahman, M.T.; Gao, C.Z.; Nie, R.J. Hypolipidemic mechanism of gypenosides via inhibition of pancreatic lipase and reduction in cholesterol micellar solubility. *Eur. Food Res. Technol.* **2016**, *242*, 305–312. [CrossRef]

32. Broeders, E.P.M.; Nascimento, E.B.M.; Havekes, B.; Brans, B.; Roumans, K.H.M.; Tailleux, A.; Schaart, G.; Kouach, M.; Charton, J.; Deprez, B.; et al. The bile acid chenodeoxycholic acid increases human brown adipose tissue activity. *Cell Metab.* **2015**, *22*, 418–426. [CrossRef] [PubMed]
33. Onal, G.; Kutlu, O.; Gozuacik, D.; Emre, S.D. Lipid droplets in health and disease. *Lipids Health Dis.* **2017**, *16*, 128. [CrossRef] [PubMed]
34. Tian, H.; Yang, C.; Yu, Y.; Yang, W.; Lu, N.; Wang, H.; Liu, F.; Wang, A.; Xu, X. Dietary cholesterol level affects growth, molting performance and ecdysteroid signal transduction in *Procambarus clarkii*. *Aquaculture* **2020**, *523*, 735198. [CrossRef]
35. Xingyong, L.; Xinglian, C.; Lijuan, D.; Tao, L.; Benlin, Y.; Dongshun, Y.; Jinliang, S.; Luxiang, W. The interaction of various factors leads to rapid degradation of chlorogenic acid in roasted coffee beans during processing. *Food Sci. China* **2021**, *42*, 7–14.
36. Josa, M.; Urizar, J.P.; Rapado, J.; Dios-Vieitez, C.; Castaneda-Hernandez, G.; Flores-Murrieta, F.; Renedo, M.J.; Troconiz, I.F. Pharmacokinetic/pharmacodynamic modeling of antipyretic and anti-inflammatory effects of naproxen in the rat. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 198–205.
37. De, A.; Singh, M.F.; Singh, V.; Ram, V.; Bisht, S. Treatment effect of L-Norvaline on the sexual performance of male rats with streptozotocin induced diabetes. *Eur. J. Pharmacol.* **2016**, *771*, 247–254. [CrossRef]
38. Priefert, H.; Rabenhorst, J.; Steinbuchel, A. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 296–314. [CrossRef]
39. Nauman, M.; Kale, R.K.; Singh, R.P. Polyphenols of *Salix aegyptiaca* modulate the activities of drug metabolizing and antioxidant enzymes, and level of lipid peroxidation. *BMC Complement. Altern. Med.* **2018**, *18*, 81. [CrossRef]
40. Zhao, D.; Sun, J.; Sun, B.; Zhao, M.; Zheng, F.; Huang, M.; Sun, X.; Li, H. Intracellular antioxidant effect of vanillin, 4-methylguaiacol and 4-ethylguaiacol: Three components in Chinese Baijiu. *RSC Adv.* **2017**, *7*, 46395–46405. [CrossRef]
41. Gutierrez-Merino, C.; Lopez-Sanchez, C.; Lagoa, R.; Samhan-Arias, A.K.; Bueno, C.; Garcia-Martinez, V. Neuroprotective actions of flavonoids. *Curr. Med. Chem.* **2011**, *18*, 1195–1212. [CrossRef] [PubMed]
42. Zheng, X.J.; Xiao, H.; Zeng, Z.; Sun, Z.W.; Lei, C.; Dong, J.Z.; Wang, Y. Composition and serum antioxidation of the main flavonoids from fermented vine tea (*Ampelopsis grossedentata*). *J. Funct. Foods* **2014**, *9*, 290–294. [CrossRef]
43. Gandhi, G.R.; Neta, M.; Sathiyabama, R.G.; Quintans, J.D.S.; Silva, A.; Araujo, A.A.D.; Narain, N.; Quintans, L.J.; Gurgel, R.Q. Flavonoids as Th1/Th2 cytokines immunomodulators: A systematic review of studies on animal models. *Phytomedicine* **2018**, *44*, 74–84. [CrossRef]
44. North, M.K.; Zotte, A.D.; Hoffman, L.C. The use of dietary flavonoids in meat production: A review. *Anim. Feed. Sci. Technol.* **2019**, *257*, 114291. [CrossRef]
45. Hu, R.Z.; He, Z.Y.; Liu, M.; Tan, J.J.; Zhang, H.F.; Hou, D.X.; He, J.H.; Wu, S.S. Dietary protocatechuic acid ameliorates inflammation and up-regulates intestinal tight junction proteins by modulating gut microbiota in LPS-challenged piglets. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 328–339. [CrossRef]
46. Kakkar, S.; Bais, S. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacol.* **2014**, *2014*, 952943. [CrossRef] [PubMed]
47. Kaewmool, C.; Kongtawelert, P.; Phitak, T.; Pothacharoen, P.; Udomruk, S. Protocatechuic acid inhibits inflammatory responses in LPS-activated BV2 microglia via regulating SIRT1/NF-kappa B pathway contributed to the suppression of microglial activation-induced PC12 cell apoptosis. *J. Neuroimmunol.* **2020**, *341*, 577164. [CrossRef]
48. Abdelmageed, M.E.; Nader, M.A.; Zaghloul, M.S. Targeting HMGB1/TLR4/NF-kappa B signaling pathway by protocatechuic acid protects against 1-arginine induced acute pancreatitis and multiple organs injury in rats. *Eur. J. Pharmacol.* **2021**, *906*, 174279. [CrossRef] [PubMed]
49. Lee, J.M.; Lim, H.S.; Chung, S.K. A short and efficient stereoselective synthesis of all four diastereomers of sphingosine. *Tetrahedron Asymmetry* **2002**, *13*, 343–347. [CrossRef]
50. Chen, J.F.; Eltzschig, H.K.; Fredholm, B.B. Adenosine receptors as drug targets—what are the challenges? *Nat. Rev. Drug Discov.* **2013**, *12*, 265–286. [CrossRef]