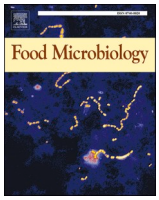
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Dual role and metabolic reprogramming: Key amino acids in marinade bidirectionally control aflatoxin B_1 biosynthesis in *Aspergillus parasiticus*

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ABSTRACT

This study revealed the effects and underlying mechanisms of key amino acids in marinade on *A. parasiticus* growth and aflatoxin B_1 (AFB_1) biosynthesis. Six key amino acids (with a concentration gradient of 0.5-8 g/L) from the marinade were selected and cultured them synchronously with *A. parasiticus* in this study. Solid/liquid culture combined with LC-MS/MS technology was used to analyse the effects of amino acids on colony growth, spore production, mycelial dry weight, and AFB_1 biosynthesis in *A. parasiticus*, and metabolomics was employed to elucidate the regulatory mechanisms of key amino acids. Results demonstrated that arginine, glycine, and lysine exhibited varying degrees of inhibitory effects. Specifically, 8 g/L lysine completely inhibited AFB_1 biosynthesis and significantly suppressed the mycelial growth. Proline, phenylalanine, and leucine remarkably promoted both the growth of *A. parasiticus* and AFB_1 production, with AFB_1 content reaching 324.50 ng/mL in the 8 g/L proline group, 3.45 times higher than in the control group. Metabolomic analysis indicated that proline synergistically enhanced AFB_1 biosynthesis by activating metabolic pathways, including the tricarboxylic acid (TCA) cycle and gibberellin metabolism. In contrast, lysine disrupted glucose metabolism and the TCA cycle, destroyed cell membrane integrity and inhibited *A. parasiticus* growth and toxin production at multiple levels. This study clarified the bidirectional regulatory effects and mechanisms of key amino acids in marinade, providing crucial technical references and theoretical foundations for controlling AFB_1 contamination in the production of marinated products by regulating marinade amino acid composition.

1. Introduction

The consumption history of marinated foods is long, and as a traditional Chinese delicacy, it occupies an important place in China's catering culture (Wu et al., 2021; Li et al., 2024). Due to their distinctive flavor and rich nutritional value, marinated foods are highly sought after by consumers. The marinade is the soul of marinated products during the marinating process, and the quality of the marinade determines the flavor and taste of the marinated products (Ai et al., 2023; Huang et al., 2022). However, factors such as the use of unqualified raw materials, improper storage, and repeated recycling during the production and use of marinade can lead to mould contamination and mycotoxin contamination (Huang et al., 2025).

Our research team has been engaged in identifying and controlling hazardous factors throughout the entire marinade and marinated product processes for a long time. Previous studies have found that *A. parasiticus* can produce aflatoxins (AFs) in stored marinade (Lin et al., 2023; Fan et al., 2023), highlighting the severe risk of AFs contamination in both marinade and marinated products. AFs are toxic secondary metabolites primarily produced by *A. flavus* and *A. parasiticus*, among which aflatoxin B_1 (AFB_1) is the most potent mycotoxin discovered to date (Huang et al., 2023; Ketney et al., 2017). AFB_1 is chemically stable and resistant to degradation, readily accumulating through metabolic and processing pathways to induce potent pathogenic, carcinogenic, and teratogenic effects (Peng et al., 2018; Zhang and Banerjee, 2020). In recent years, AFB_1 contamination has posed an extremely serious threat to human and animal health and also caused significant economic losses to the food industry (Kinyoro and Kaale, 2024; Gong et al., 2024). Consequently, AFB_1 contamination and its generation mechanisms during food processing have long been a concern for researchers, and

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AFB_1 green prevention and control are urgent bottleneck problems requiring breakthroughs in human food safety and health.

Interestingly, no residual AFB_1 was detected in the repeatedly used marinade (Chen et al., 2020), suggesting that the marinade's unique nutritional environment may suppress toxin production in *A. parasiticus*. It is well known that the growth of *A. parasiticus* and the synthesis of AFs are influenced by multiple factors, such as temperature, moisture, pH, and nutritional status (Gourama and Bullerman, 1995; Peromingo et al., 2016). Among these factors, the quality and quantity of nitrogen sources play a particularly significant role in the growth and differentiation of *Aspergillus* species. Among nitrogen sources, amino acids exert a specific influence on the growth and toxin production of *A. flavus* and *A. parasiticus* (Hashem et al., 2015; Wang et al., 2017). Preliminary research, monitoring the changes in nutrient composition of marinade during repeated recycling procedures, found significant changes in amino acid composition (Wu et al., 2024). As indispensable substances in microbial life, amino acids play key roles in *A. parasiticus* growth and AFs synthesis, and their roles have increasingly attracted the attention of researchers (Liu et al., 2016). Reddy et al. (1979) reported that asparagine is an essential nutrient for *A. parasiticus* to synthesise AFs, and the effects of alanine and aspartic acid on AFs synthesis can be equivalent to asparagine. Mateles and Adye (1965) reported that methionine, phenylalanine, tyrosine, and tryptophan are involved in regulating AFs synthesis. Wilkinson et al. (2007) also confirmed that tyrosine can stimulate the synthesis of AFs in *A. flavus*, while tryptophan inhibits the synthesis of AFs.

Understanding the effects of amino acids on *A. parasiticus* growth and AFs synthesis is crucial for gaining deeper insight into its biological characteristics and exploring the mechanisms of AFs synthesis (Daryanavard and Jarrah, 2019). Such knowledge is particularly valuable for developing targeted strategies to control AFB_1 contamination in marinade-based food systems. By identifying amino acids that inhibit toxin production, we can propose practical approaches to reformulate or adjust marinade compositions, thereby enhancing the safety of marinated products. Thus, this study selected *A. parasiticus* isolated from stored marinade by our research group as the subject, and selected key amino acids during the marinade recycling process as the culture substrate (nitrogen source) to analyse the growth and toxin production of *A. parasiticus* under different amino acid concentrations. Metabolomics was used to reveal changes in the metabolite composition of *A. parasiticus* in response to target amino acids. The research results not only provide technical guidance for preventing AFs contamination in marinade but also offer a practical theoretical basis for ensuring the quality and safety of marinated products through marinade formulation management.

2. Materials and methods

2.1. Materials and chemicals

A. parasiticus was screened and isolated from marinade in our laboratory. Proline, glycine, leucine, arginine, phenylalanine, and lysine were supplied by Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China). Potato Dextrose Broth (PDB) medium, Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Methanol and acetonitrile were supplied by Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). AFB_1 and $^{13}C_{17}$-AFB_1 were purchased from TMRM Quality Inspection Technology Co., Ltd. (Changzhou, China). All other chemical reagents were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Activation of A. parasiticus and preparation of spore suspensions

Following the method of Fan et al. (2023), the preserved *A. parasiticus* was inoculated into PDA medium and activated at 28 °C for 7 days. Spores were washed with sterile physiological saline containing 0.5% Tween-80 and counted under a microscope using a hemocytometer to prepare into a spore suspension at a concentration of 10^6 CFU/mL. The suspension was stored at 4 °C for future use.

2.3. Determination of colony diameter and spore production capacity of A. parasiticus

Proline, glycine, leucine, arginine, phenylalanine, and lysine were added to the solid minimal medium (30 g sucrose, 0.5 g KCl, 0.5 g $MgSO_4 \bullet 7H_2O$, 0.01 g $FeSO_4 \bullet 7H_2O$, 1 g K_2HPO_4, 15 g/L agar powder, adjusted to 1 L with water) at concentrations of 0.5, 1, 2, 4, and 8 g/L 5 μL of *A. parasiticus* spore suspension was taken and placed in the center of solid minimal medium containing different amino acids, with no added amino acids as the control. The culture dish was placed upside down and incubated at 28 °C for 7 days in a constant-temperature incubator (IS-AX, Suzhou Jiemei Electronics Co., Ltd., China). The morphology of the colonies was observed, and their diameters were measured using the cross method. At the same time, 10 mL of sterile physiological saline containing 0.05% Tween-80 was added to the culture dish. The culture washed, filtered through four layers of sterile gauze, and spore counts were performed using a hemocytometer. Each group included three replicates.

2.4. Determination of dry weight of A. parasiticus mycelium

Proline, glycine, leucine, arginine, phenylalanine, and lysine were added to the liquid minimal medium (30 g sucrose, 0.5 g KCl, 0.5 g $MgSO_4 \bullet 7H_2O$, 0.01 g $FeSO_4 \bullet 7H_2O$, 1 g K_2HPO_4, adjusted to 1 L with water) at the same concentrations as 2.3. 100 μL of *A. parasiticus* spore suspension was inoculated into liquid minimal medium containing different amino acids, with no added amino acids as the control. After shaking and culturing at 28 °C and 200 r/min for 7 days, the mycelium was filtered and dried (80 °C, 6 h). The mycelium dry weight was obtained when a constant weight was reached (Li et al., 2022).

2.5. Determination of AFB_1 content

In this experiment, 0.9 mL of *A. parasiticus* spore suspension was inoculated into PDB medium containing six different amino acids at concentrations of 0.5, 1, 2, 4, and 8 g/L. The conical flask with inoculated PDB medium was placed in a shaking culture at 28 °C and 200 r/min for 7 days. Vortex the mycelium with acetonitrile for 15 min, and transfer 4 mL of the lower solution into a 10 mL centrifuge tube. The centrifuge tube solution was slowly blown to near dryness with nitrogen at 80 °C, then dissolved in methanol and vortexed for 30 s to dissolve the residue. The above solution was filtered through a 0.22 μm organic filter membrane, and 30 μL of internal standard solution ($^{13}C_{17}$-AFB_1) was added to the injection bottle for quantitative detection using LC-MS/MS (Fan et al., 2023).

2.6. Extraction and detection of metabolites from A. parasiticus

The spore suspension of *A. parasiticus* (10^6 CFU/mL) was inoculated into liquid minimal medium containing 8 g/L proline and lysine, and incubated at 28 °C with shaking at 200 r/min for 72 h. A Control group without amino acid supplementation was also set up simultaneously. The mycelium of *A. parasiticus* from each experimental group was collected, filtered through gauze, and then quickly rinsed 2-3 times with pre-cooled PBS. The cleaned mycelium were centrifuged (4 °C, 4000 rpm) for 5 min, and the collected mycelium were placed in 2 mL centrifuge tubes and stored at −80 °C.

0.5 g of the *A. parasiticus* mycelium was accurately weighed and placed in a 2 mL centrifuge tube. 1000 μL of 50% methanol water was added, vortexed for 30 s, and then centrifuged (12000 rpm, 4 °C) for 10 min. The supernatant was concentrated and dried, then dissolved in 300 μL of 2-chloro-L-phenylalanine (4 mg/kg) solution prepared in 50% methanol-water. The supernatant was filtered through a 0.22 μm

membrane and analysed using LC-MS/MS (liquid chromatography, Vanquish, Thermo, US; mass spectrometer, Orbitrap Exploris 120, Thermo, US). The chromatographic conditions, mass spectrometry conditions, and metabolomic data processing and analysis were set according to the method of Lu et al. (2024).

Peak detection, retention time correction and alignment were first performed on the mass spectrometry data to obtain a quantitative list of metabolites. The key parameters for metabolomic data analysis were set as follows: smooth bandwidth (bw = 2), mass deviation margin (ppm = 15), peak width range (5 - 30 s), mass window (mzwid = 0.015), and minimum mass difference (mzdiff = 0.01). Then, the metabolites were identified by accuracy mass and MS/MS data which were matched with HMDB, massbank, KEGG, LipidMaps, mzcloud and the metabolite database bulid by Panomix Biomedical Tech Co., Ltd. (Shuzhou, China).

2.7. Statistical analysis

Each assay was performed in triplicate, with results expressed as mean ± standard deviation (SD). Data analysis was performed using SPSS 22.0 software (IBM, Chicago, USA) and OriginPro 2024 software (OriginLab, Massachusetts, USA). A p-value <0.05 was considered statistically significant for determining differences between groups. For metabolomics data, orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to maximize inter-group differences. Differential metabolites were screened based on variable importance in projection (VIP) > 1 and P-value <0.05 from Student's t-test. KEGG pathway enrichment analysis was used to determine metabolic pathways significantly affected by amino acid stress, mainly by using hypergeometric distribution enrichment analysis method to enrich functional pathways and topological analysis of metabolites.

3. Results and discussion

3.1. Effects of different concentrations of amino acids on the colony morphology and diameter of A. parasiticus

Changes in the colony morphology of *Aspergillus parasiticus* under different concentrations of six amino acids are shown in Fig. 1. There is a significant difference in colony morphology of *A. parasiticus* between cultures with and without added amino acids, with almost no obvious

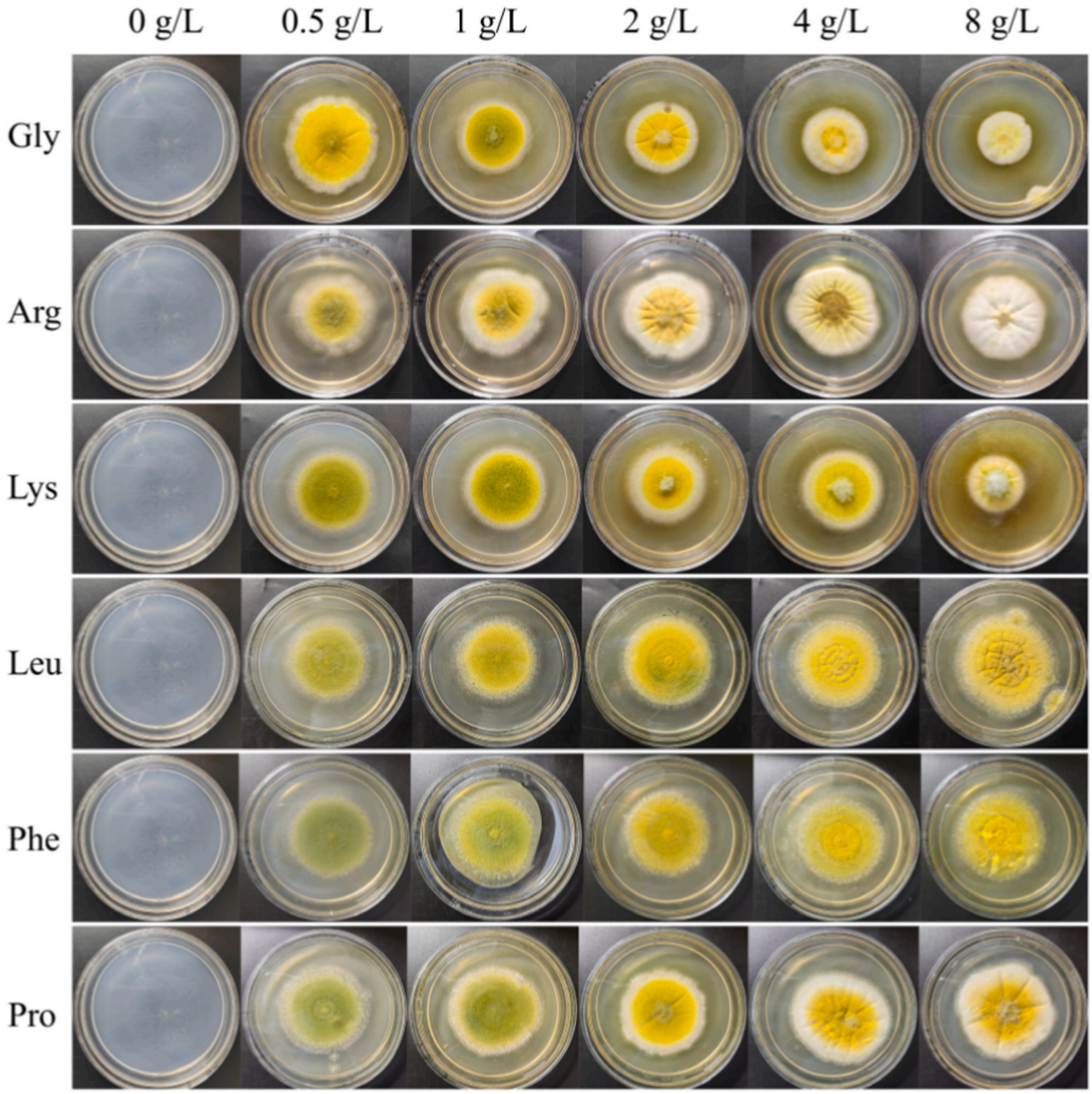


Fig. 1. Changes in the colony morphology of *A. parasiticus* under different concentrations of six amino acids stress after 7 days of culture on solid minimal medium.

hyphae visible without added amino acids. Such an outcome may result from amino acids being essential for microbial growth; a lack of amino acids prevents normal protein synthesis, thereby inhibiting mycelial growth (Marroquin-Guzman et al., 2018). The addition of lysine can significantly inhibit the increase in colony diameter and slow the growth of *A. parasiticus*, as observed in overall colony morphology. Specifically, 8 g/L lysine can cause *A. parasiticus* spores to appear sparse, with reduced sporulation capacity and abnormal physiological morphology, possibly involving amino acids metabolic pathways or toxic effects. The colony color of *A. parasiticus* in the glycine and arginine groups gradually shifted from yellow-green to white with increasing concentration, which may be due to glycine and arginine inhibiting the expression of key enzymes involved in AFs synthesis and oxidative stress response genes, thereby disrupting secondary metabolism and reducing the accumulation of yellow pigment (Amare and Keller, 2014). Additionally, the colony color of *A. parasiticus* gradually changed from green to yellow. The colony edge appeared white under the action of proline, leucine, and phenylalanine, which may be because these amino acids promote mycelial expansion, leading to a yellow color in the colony center (Cárdenas and Hansberg, 1984). In contrast, the white edge may represent newly formed mycelium.

The effects of six amino acids at different addition concentrations on the diameter of *A. parasiticus* colonies are shown in Fig. 2. Compared with the control group, the colony diameter of *A. parasiticus* in the arginine and glycine groups gradually decreased with increasing concentration. The colony diameter decreased to 4.12 cm and 5.5 cm at 8 g/L, respectively. Previous studies have confirmed that arginine can be converted into polyamines through arginine decarboxylase, and excessive polyamines can disrupt membrane potential or induce DNA damage (Majumdar et al., 2018), leading to inhibition of mycelium growth. The colony diameters of *A. parasiticus* are 6.65 cm and 6.52 cm in lysine group with the addition concentrations of 0.5 g/L and 1 g/L, respectively, significantly higher than those in other treatment groups, indicating that low concentrations of lysine can promote the growth of *A. parasiticus*. When the concentration of lysine was increased to 2 g/L, the colony diameter decreased significantly (from 6.52 cm to 5.05 cm). In comparison, when the concentration of lysine was 8 g/L, the colony diameter decreased to 3.63 cm. Such an outcome may result from lysine inhibiting the fungal lysine biosynthesis pathway via negative feedback, leading to a shortage of key intermediate metabolites, which in turn, affects the tricarboxylic acid cycle and energy metabolism, thereby inhibited *A. parasiticus* growth (Gabriel and Rychłowski, 2017). Compared with the control group, the colony diameter of *A. parasiticus* in the proline, leucine, and phenylalanine groups gradually increased with increasing concentration, reaching 7.23 cm at 8 g/L proline. This may be attributed to the correlation between proline and the

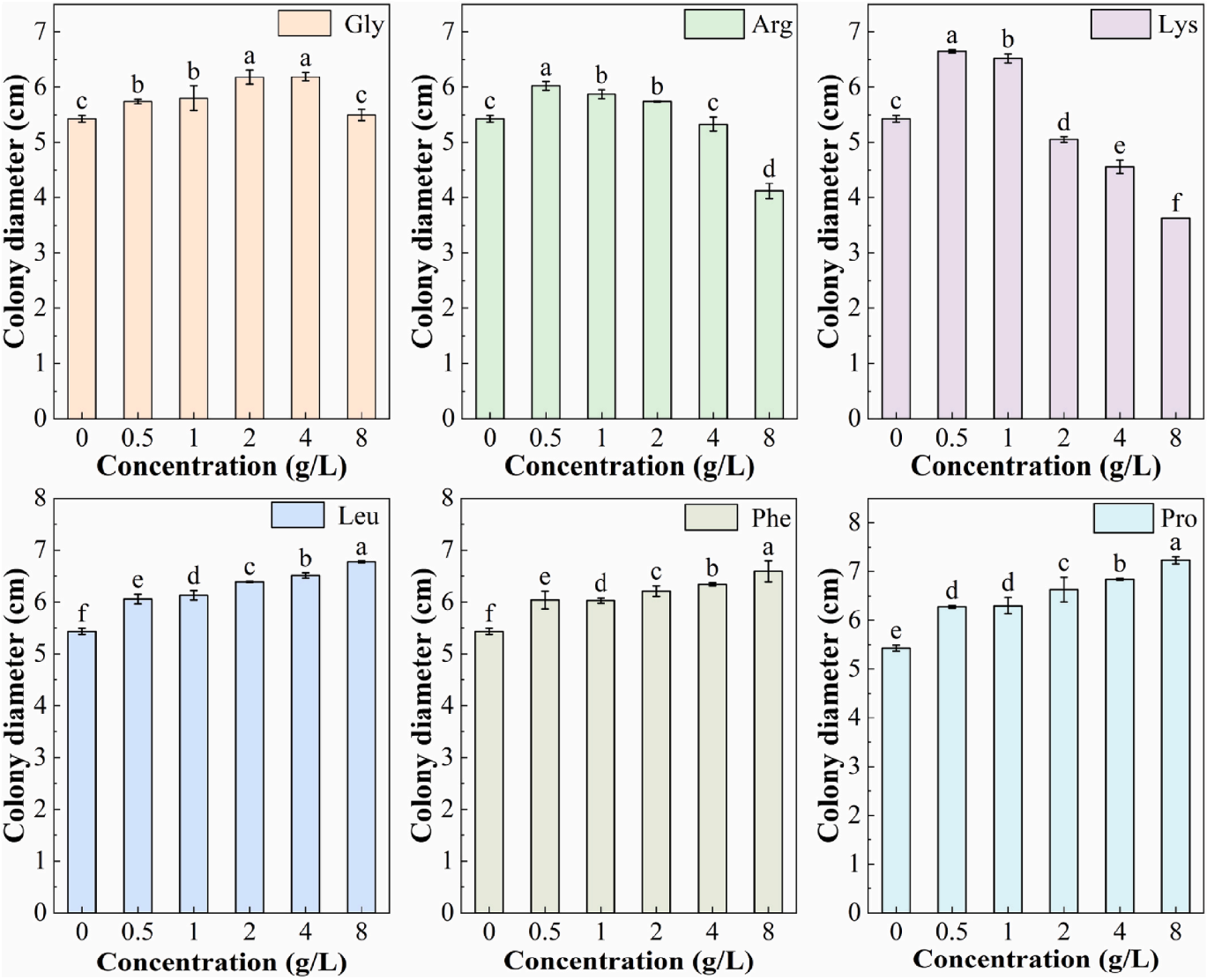


Fig. 2. Changes in the colony diameter of *A. parasiticus* under different concentrations of six amino acids stress after 7 days of culture on solid minimal medium.

mitochondrial proline catabolic pathway in fungi, which is closely linked to the fungal survival (Zhang et al., 2024).

3.2. Effects of different concentrations of amino acids on spore production of *A. parasiticus*

Changes in the spore production of *A. parasiticus* under different concentrations of six amino acids are shown in Fig. 3. Compared with the control group, the spore production of *A. parasiticus* gradually decreased with increasing concentration in the lysine group, but significantly increased with increasing concentrations in the leucine and phenylalanine groups. The spore production under the action of 2 g/L lysine, leucine, and phenylalanine was 1.65×10^7 cfu/mL, 3.93×10^7 cfu/mL, and 5.40×10^7 cfu/mL, respectively. When the amino acids concentration increased to 8 g/L, the spore production of the lysine group was only 0.20×10^7 cfu/mL. In contrast, the spore production of the leucine and phenylalanine groups increased to 10.00×10^7 cfu/mL and 4.60×10^7, respectively. 50 and 23 times higher than the lysine group. These results indicate that only lysine, among essential amino acids, can significantly inhibit the spore production in of *A. parasiticus*. Notably, leucine and phenylalanine can significantly promote the spore production in of *A. parasiticus*. It is posited that fungal spore formation requires specific nitrogen-source signalling regulation (e.g., *Area* transcription factor), and lysine may suppress the expression of sporulation-related genes by interfering with the nitrogen metabolism regulatory network mediated by GATA factors (Chudzicka-Ormaniec et al., 2019).

There are significant differences in the effects of non-essential amino acids (proline, glycine, and arginine) on the spore production of *A. parasiticus*. Among them, the spore production of *A. parasiticus* gradually increased with the increase of proline concentration, from 5.83×10^7 cfu/mL to 13.05×10^7 cfu/mL. Spore production gradually decreased with increasing concentrations under the stress of glycine and arginine, resulting in 2.95×10^7 and 0.60×10^7 cfu/mL at 8 g/L, respectively. The significant differences in the effects of proline, glycine, and arginine on the spore production of *A. parasiticu* may be due to: 1) glycine is a key substrate in the folate cycle (one carbon metabolism), and excessive glycine may disrupt the balance of methyl donors (Locasale, 2013); 2) arginine is converted into polyamines by arginine decarboxylase, and excessive polyamines can increase mitochondrial membrane permeability and ROS accumulation, thereby inhibiting sporulation (Zahedi et al., 2022); 3) proline can act as a compatible solute to alleviate osmotic stress and activate related regulatory pathways, thereby promoting the expression of stress response genes related to sporulation (Duran et al., 2010).

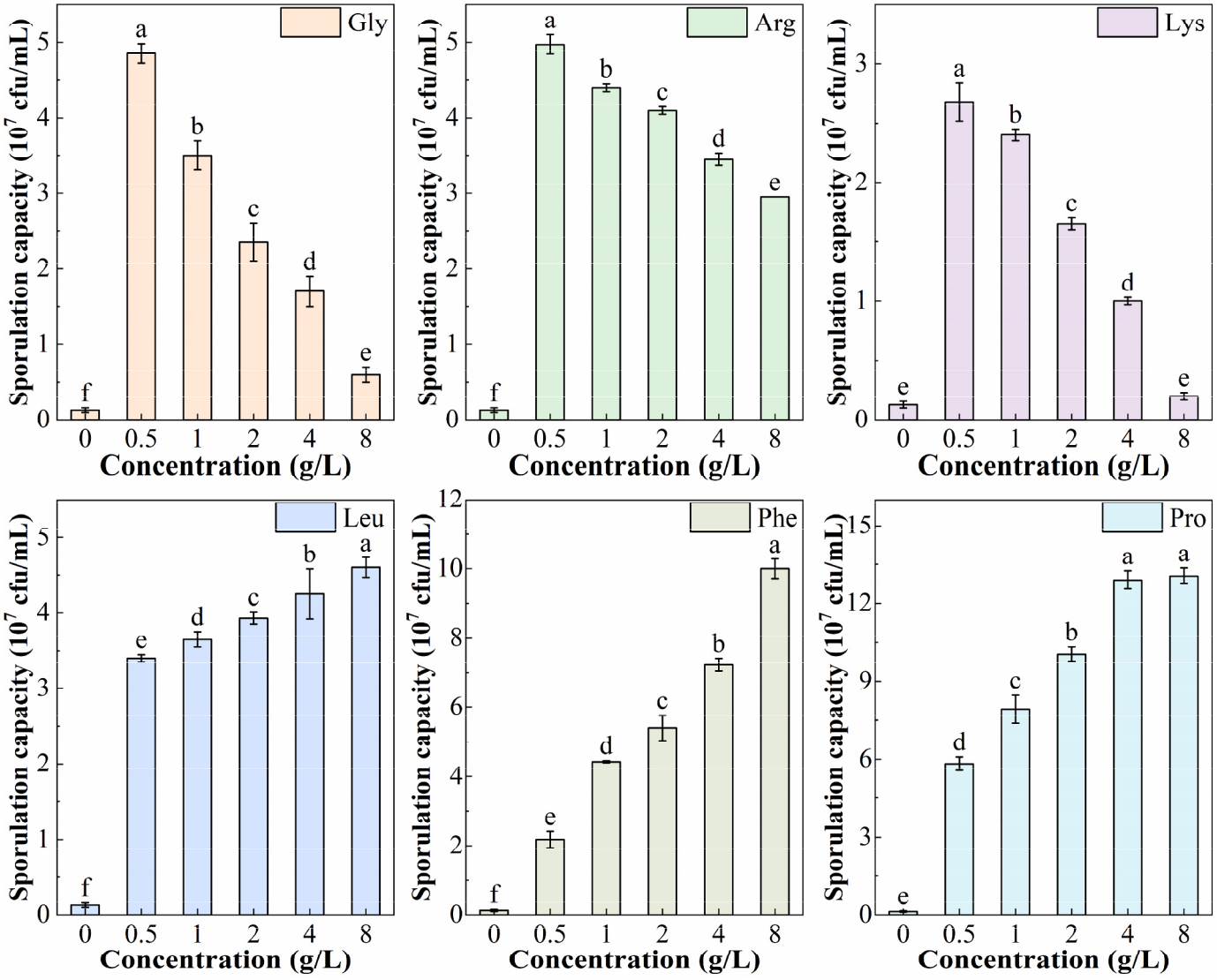


Fig. 3. Changes in the spore production of *A. parasiticus* under different concentrations of six amino acids stress after 7 days of culture on solid minimal medium.

3.3. Effects of different concentrations of amino acids on dry weight of *A. parasiticus* mycelium

Changes in the dry weight of *A. parasiticus* mycelium under different concentrations of six amino acids are shown in Fig. 4. All three essential amino acids (leucine, phenylalanine, and lysine) were found to promote the mycelium growth of *A. parasiticus*, and the dry weight of mycelium increased with increasing amino acid concentration. By comparison, phenylalanine had a greater impact on the mycelial dry weight than leucine. In contrast, lysine had a less substantial impact on the dry weight of *A. parasiticus* mycelium. Specifically, phenylalanine can significantly promoted the growth of *A. parasiticus* mycelium, with a dry weight of 132.57 mg at 8 g/L. This outcome is similar to that of Liu et al. (2020), who reported that phenylalanine significantly promoted the growth of *A. flavus* mycelium. Three essential amino acids may promote mycelial growth by activating the TORC1 pathway via the SPS sensors, thereby stimulating ribosomal biosynthesis and translation initiation, which in turn, promote mycelial growth (Kim and Guan, 2011).

Compared with the control group, the dry weight of *A. parasiticus* mycelium gradually increased with increasing concentration of non-essential amino acids (proline, glycine, and arginine) added. Meanwhile, the concentration of amino acids is positively correlated with the dry weight of *A. parasiticus* mycelium, indicating that proline, glycine, and arginine can significantly promote the growth of *A. parasiticus* mycelium. In this study, we found that glycine and arginine had relatively minor effects on the dry weight of *A. parasiticus* mycelium, with the arginine group showing a complex trend: first increasing, then decreasing, then increasing again. Meanwhile, proline had the most significant effect on the dry weight of *A. parasiticus* mycelium, with 8 g/L proline increasing in mycelial dry weight to 145.07 mg. These data are consistent with previous reports that proline can significantly increase colony diameter. It is speculated that high concentrations of proline may not only provide sufficient nitrogen for the growth of *A. parasiticus*, but also stimulate its energy metabolism and protein synthesis and promote mycelial growth (Silao et al., 2019; Wu et al., 2017).

3.4. Effects of different concentrations of amino acids on AFB_1 synthesis

Changes in the synthesis of AFB_1 by *A. parasiticus* under different concentrations of six amino acids are shown in Fig. 5. Compared with the control group, the AFB_1 content in the lysine, arginine, and glycine groups gradually decreased with increasing concentration. 8 g/L lysine completely inhibited AFB_1 synthesis, while the inhibition rates of 8 g/L arginine and 8 g/L glycine on AFB_1 synthesis were 82.27% and 60.75%, respectively. The significant differences in the effects of lysine, arginine, and glycine on AFB_1 synthesis may be due to: 1) high concentrations of

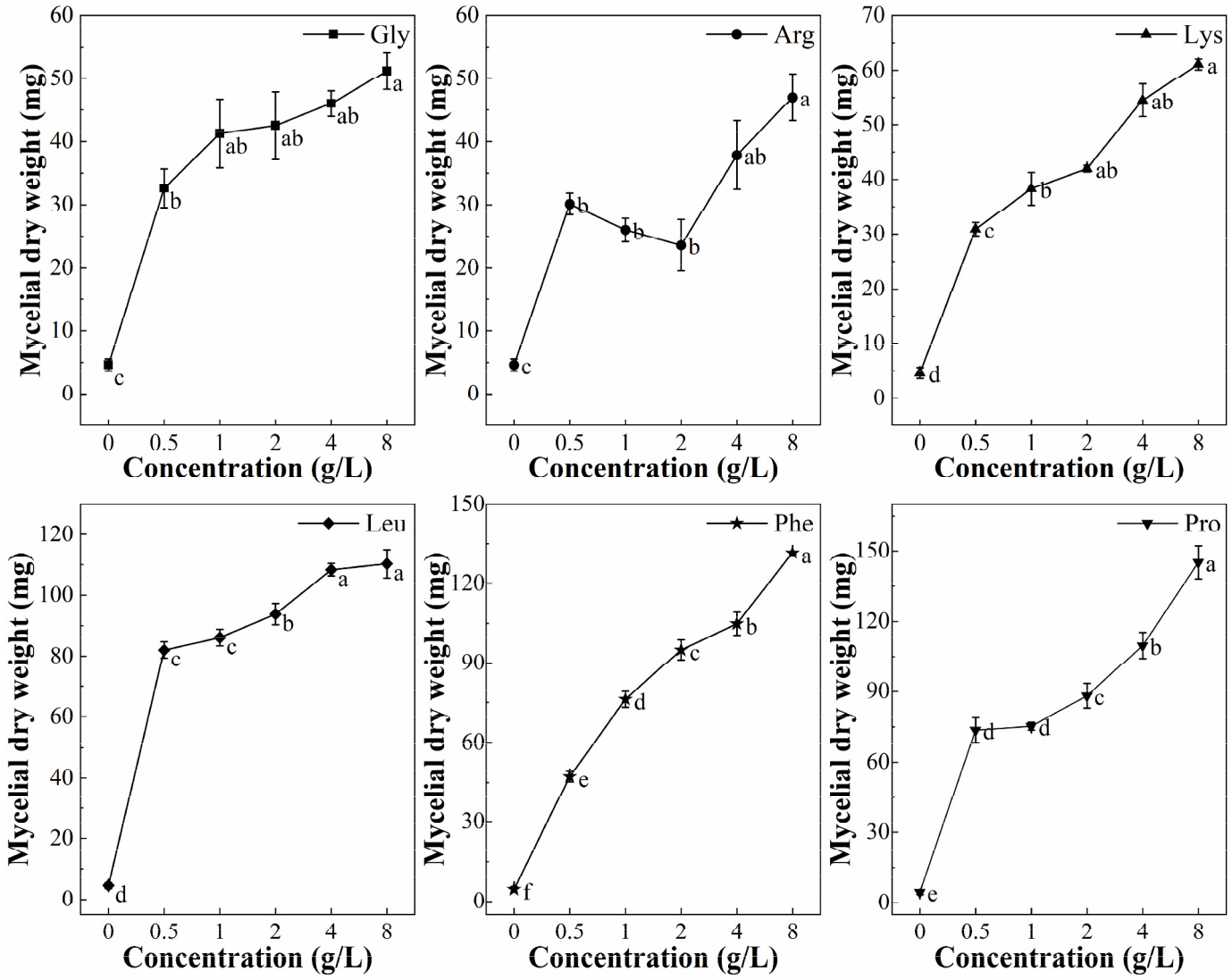


Fig. 4. Changes in the mycelial dry weight of *A. parasiticus* under different concentrations of six amino acids stress after 7 days of culture in liquid minimal medium.

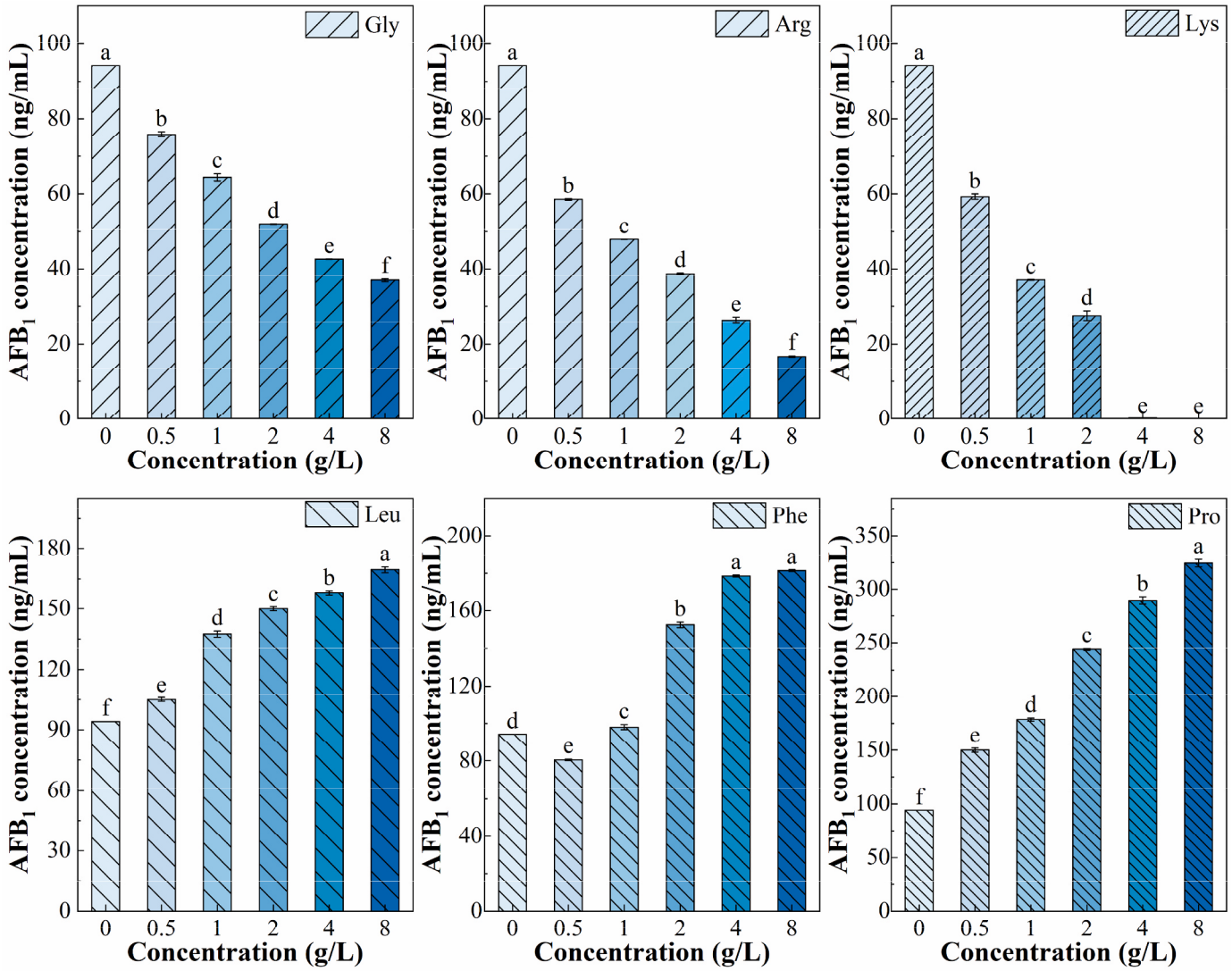


Fig. 5. Changes in the AFB_1 synthesis of *A. parasiticus* under different concentrations of six amino acids stress after 7 days of culture in PDB medium.

lysine affecting cell nitrogen metabolism, thereby affecting the carbon-nitrogen balance required for *A. parasiticus* to synthesise AFB_1 (Cybis and Weglenski, 1969); 2) arginine can be converted into polyamines in fungi, which have polyamine toxicity and cause oxidative stress, thereby inhibiting the normal metabolic activity of *A. parasiticus* (Sannazzaro et al., 2004); 3) glycine may affect the epigenetic regulation of toxin synthesis genes, thereby inhibiting the expression of genes such as *AflR* and *AflS* (Wang et al., 2024).

In this study, the AFB_1 content in the proline, phenylalanine, and leucine groups gradually increased with increasing concentration. In particular, the AFB_1 content in the 8 g/L proline group was as high as 324.50 ng/mL. In contrast, the AFB_1 content in the 8 g/L phenylalanine and leucine groups reached 181.50 ng/mL and 169.5 ng/mL, respectively, levels equivalent to 1.80-3.45 times those of the control group. Research has shown that microorganisms often metabolise phenylalanine and leucine to produce pyruvate and acetyl-CoA, key metabolic intermediates that can significantly promote the biosynthesis of secondary metabolites (Peng et al., 2015). Additionally, the proline metabolic pathway in fungi plays an important role, as it can effectively activate the biosynthetic mechanism of fungal secondary metabolites via a mitochondrial-specific catabolic pathway. This metabolic characteristic is significantly correlated with the ability to synthesise mycotoxins and may be an important factor affecting its potential to produce toxin (Cheng et al., 2013). The effects of various amino acids on AFB_1 synthesis in this study are similar to the results of Liu et al. (2020), indicating that appropriate levels of amino acids promote or inhibit the synthesis of AFB_1 by *A. parasiticus*. However, the detailed regulatory mechanism still needs to be further studied using metabolomics.

3.5. Statistical analysis of differential metabolite profiles in A. parasiticus under lysine and proline stress

Based on the above research results, proline has a significant promoting effect on the growth and AFB_1 synthesis of *A. parasiticus*, whereas lysine exhibits the most potent inhibitory effect on both. Therefore, proline and lysine were selected for treating *A. parasiticus* and for metabolite differential analysis. The total ion chromatograms (TIC) of metabolites in proline, lysine, and the control group under positive and negative ion modes are shown in Fig. 6. In this study, a total of 1624 metabolites were identified as *A. parasiticus* in each experimental group through comparative analysis. The OPLS-DA model was further used for inter-group pairing comparison analysis, and the secondary differentially expressed metabolites between groups were screened using VIP >1 and $P < 0.05$ as thresholds. The number of differential metabolites between the proline and control groups was 946, with 480 significantly upregulated and 466 significantly downregulated. The number of

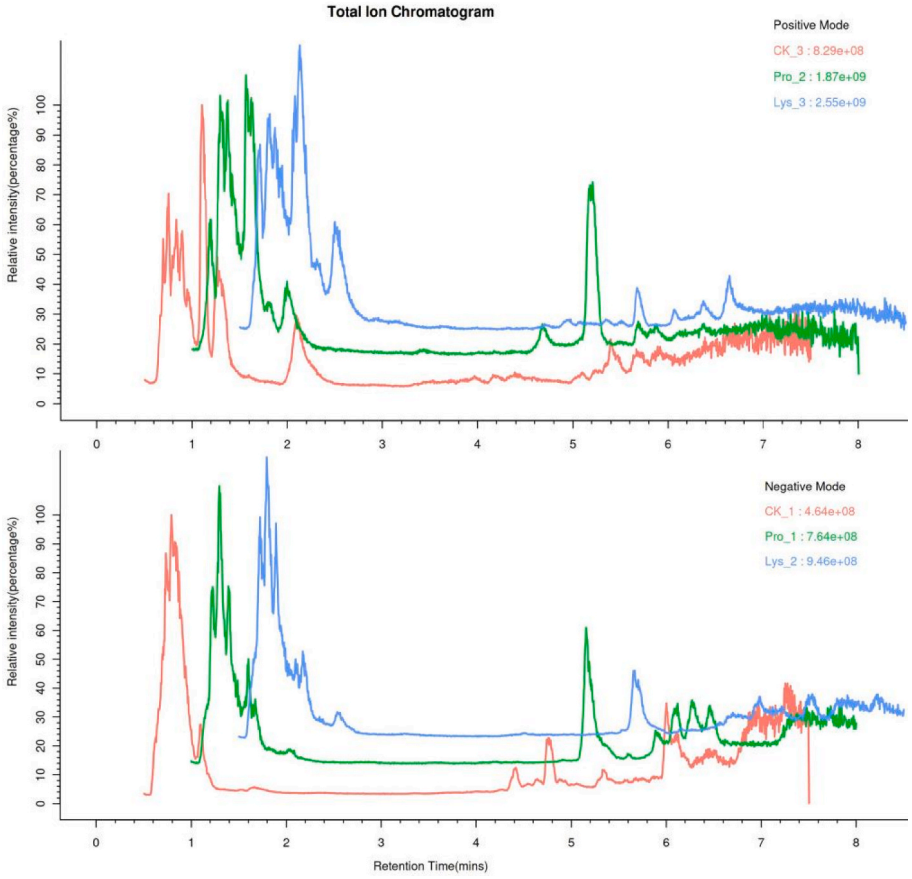


Fig. 6. Total ion flow diagram of metabolites extracted from *A. parasiticus* mycelium in positive (A) and negative (B) ion modes.

differential metabolites between the lysine and control groups was 1047, with 544 significantly upregulated and 503 significantly downregulated. The number of differentially expressed metabolites between the lysine and proline groups was 741, with 388 significantly upregulated and 353 significantly downregulated (Table 1).

3.6. Differential analysis of metabolites in A. parasiticus under lysine and proline stress

Cluster heatmap analysis was conducted on differentially expressed metabolites in *A. parasiticus* under proline and lysine, where the gradient colors represent the magnitude of quantitative values, with red colors indicating upregulation and blue colors indicating downregulation (Fig. 7). Compared with the control group, L-proline was significantly upregulated in the proline group, as prolin was the treatment factor in this study, which met the expectations of experimental design and also reflected the absorption and utilisation of proline by *A. parasiticus*. Wu et al. (2017) demonstrated that proline is not only an important osmoprotectant but also serves as a carbon/nitrogen source and a regulator of redox balance, playing multiple roles in fungal stress adaptation and growth. The high accumulation of L-proline in this study provided sufficient nutrients for *A. parasiticus*, promoting mycelial growth and spore differentiation. Furthermore, proline can participate in the nitrogen metabolism cycle of fungi by being converted to glutamate, supplying a nitrogen source for toxin synthesis (Silao et al., 2019). This result confirms the core role of proline as a key nutritional factor in the growth and toxin production of *A. Parasiticus* at the metabolic level.

Methyl gibberellin A9 is an important member of the gibberellin family, which are plant hormone analogues. It has also been found in fungi and plays an important regulatory role in fungal growth and development. Studies have indicated that some fungi can synthesise gibberellins to regulate their own growth and development, and exogenous addition of gibberellins significantly promote mycelial elongation and spore germination in *A. flavus* (Bilal et al., 2018; Hamayun et al., 2009). In addition, gibberellins can enhance the expansion ability of fungal hyphae by regulating the activity of enzymes involved in fungal cell wall synthesis (Tudzynski, 2005). In this study, the significant upregulation of methyl gibberellin A9 revealed that proline may promote cell elongation and hyphal growth in *A. parasiticus* by activating the gibberellin metabolic pathway, which is highly consistent with the phenotype observation of enlarged colony diameter of *A. parasiticus* in the proline group.

Citric acid, as a key intermediate in the tricarboxylic acid (TCA) cycle, is the core pathway of fungal energy metabolism, and its metabolic activity directly reflects the energy supply status of cells (Zhang et al., 2019). Citric acid was significantly upregulated in the proline group, indicating that proline treatment significantly activated the energy metabolism pathways in the *A. parasiticus*. Furthermore, the synthesis of aflatoxins requires a large amount of energy, and enhanced TCA cycle activity can provide sufficient ATP and NADPH for AFB_1 synthesis

Table 1
Number of differentially expressed metabolites in *A. parasiticus* identified by pairwise comparisons among the control, proline (8 g/L), and lysine (8 g/L) groups based on OPLS-DA analysis (VIP >1, $p < 0.05$).

Comparison	Total Metabolites Count	Upregulated Differential Metabolites Count	Downregulated Differential Metabolites Count	Total Count of Differential Metabolites
Pro vs CK	1624	480	466	946
Lys vs CK	1624	544	503	1047
Lys vs Pro	1624	388	353	741

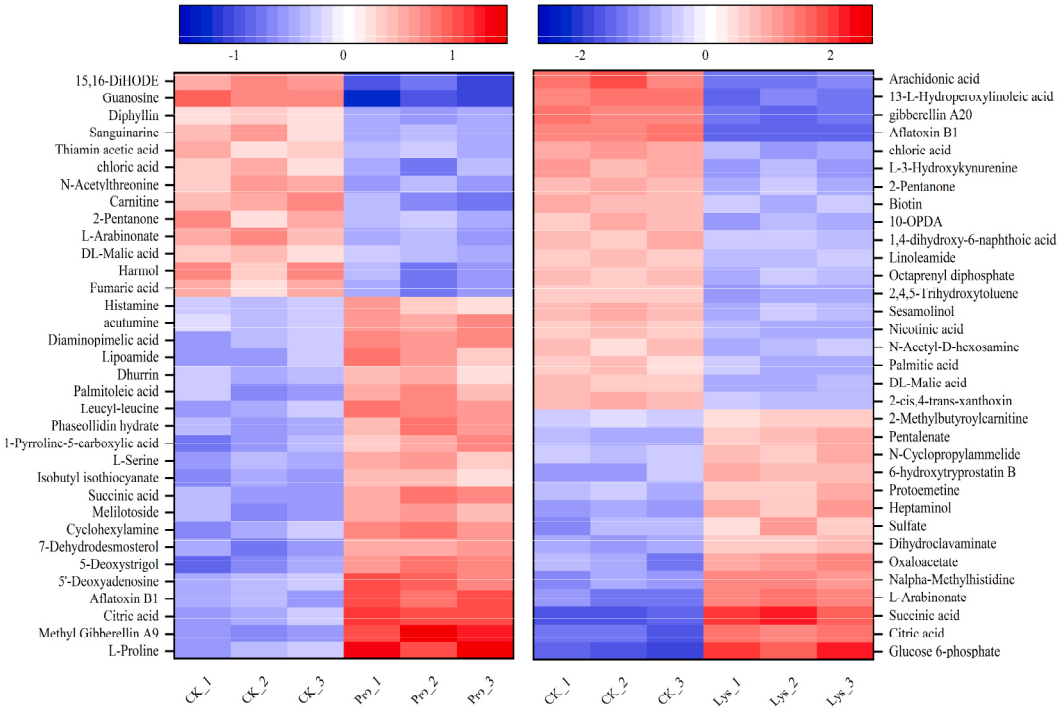


Fig. 7. Heatmap of differential metabolites in *A. parasiticus* under proline and lysine stress. Red colors indicate upregulation and blue colors indicate downregulation (VIP >1, P < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Falade et al., 2018). Meanwhile, citric acid serves as an important precursor for lipid synthesis, and its accumulation can promote the synthesis of fungal cell membranes, providing a structural basis for hyphal growth and spore development (Martins et al., 2025). The significant upregulation of citric acid in this study perfectly explains the dual phenotype of vigorous growth and increased toxin production of the *A. parasiticus* proline group, revealing that activated energy metabolism is one of the key mechanisms through which proline exerts its effects. Recent studies have elucidated the mechanism of AFB_1 synthesis, showing that the *aflR* and *aflS* genes are core regulatory factors, and that their expression levels are positively correlated with AFB_1 production (Khan et al., 2021). The significant upregulation of AFB_1 in this study is highly consistent with this mechanism, further confirming that proline promotes *A. parasiticus* toxin production by activating the toxin synthesis pathways. Additionally, Peng et al. (2024) found that energy metabolism status is a key regulator of AFB_1 synthesis, as evidenced by a significant increase in AFB_1 synthesis when fungi are in an energy-abundance state, consistent with the upregulation of energy-related metabolites, such as citric acid, observed in this study.

5′-Deoxyadenosine, a derivative of adenosine, not only participates in purine metabolism and methyl transfer reactions, but also in coenzyme synthesis and signal transduction (Dunken et al., 2024; Li et al., 2023). Gerke et al. (2012) demonstrated that 5′-deoxyadenosine serves as an important precursor for the synthesis of S-adenosylmethionine (SAM), the primary methyl donor in fungi, which participates in DNA methylation and secondary metabolite synthesis. In *A. flavus*, a sufficient supply of SAM can promote the expression of genes involved in AFB_1 synthesis (Jia et al., 2024). The significant upregulation of 5′-deoxyadenosine in this study suggests that proline treatment may provide conditions for epigenetic regulation of AFB_1 synthesis-related genes by increasing the supply of methyl donors, thereby promoting AFB_1 synthesis.

Guanosine is an important intermediate product in purine nucleotide metabolism, and guanosine and its derivatives mainly participate in RNA synthesis, energy metabolism, and signal transduction in fungi (Nong et al., 2024). The hydrolysis of guanosine triphosphate (GTP) is a key step in activating G protein signalling, and guanosine deficiency can inhibit GTP regeneration, thereby affecting the sustained activation of specific G protein signalling pathways (Agogo-Mawuli et al., 2025). Interestingly, the inactivation of specific G proteins or their coupled receptors actually promotes AFB_1 synthesis in *A. flavus* and *A. parasiticus* (Qiu et al., 2021; Souza et al., 2013). The significant downregulation of guanosine in this study may play a key role in promoting AFB_1 synthesis by selectively regulating the balance among different G protein signalling pathways, and this regulatory mechanism warrants further in-depth investigation.

5S,8R-DiHODE is a specific oxylipid metabolite generated by linoleic acid catalysed by lipoxygenase, belonging to the negative regulatory signalling molecule in mycotoxin synthesis (Liu et al., 2023). These specific oxylipid metabolites can competitively bind to the *GprC/GprD* receptors in *A. flavus*, block activation of the G protein cAMP/PKA pathway, inhibit transcription of the toxin-producing core gene *aflR*, and reduce the fluidity of fungal cell membranes, thereby inhibiting mycelial expansion (Liu et al., 2023; Gessler et al., 2017). Previous studies confirmed that exogenous addition of 10 μmol/L 5S,8R-DiHODE reduced AFB_1 production by 78% in *A. flavus* and significantly inhibited colony diameter expansion (Brodhun and Feussner, 2011). In this study, 15,16-DiHODE was significantly downregulated in the proline group and negatively correlated with AFB_1 synthesis. This result is mutually validated with the phenotype of proline group-promoting AFB_1 synthesis and mycelial growth, indicating that proline promotes *A. parasiticus* growth and toxin production by reducing the levels of negatively regulated oxylipins, thereby relieving their inhibition of the G protein cAMP/PKA pathway. Consequently, 15,16-DiHODE can be used as a key downregulated biomarker for proline to promote the growth and AFB_1 synthesis of *A. parasiticus*.

Compared with the control group, AFB_1 expression was significantly downregulated in the lysine group, directly verifying the above experimental conclusion that “lysine inhibits AFB_1 production”. It is posited that lysine may inhibit the transcription of regulatory genes such as *aflC*, *aflD*, and *aflR*, thereby suppressing AFB_1 synthesis. Concurrently, gibberellin A20 was significantly downregulated in the lysine group,

which is highly consistent with the above phenotype, "lysine inhibits mycelial growth", indicating that lysine may suppress gibberellin accumulation by inhibiting key steps in the gibberellin biosynthesis pathway, thereby inhibiting mycelial expansion and spore differentiation (Tudzynski, 2005). Thus, the significant downregulation of gibberellin A20 and AFB_1 serves as an important biomarker for lysine inhibition of *A. parasiticus* growth and AFB_1 synthesis.

Glucose-6-phosphate, citric acid, and succinic acid are metabolites produced during the energy metabolism process. Among these, glucose-6-phosphate serves as the starting substrate for glycolysis and a key intermediate product of the pentose phosphate pathway. Glucose-6-phosphate not only provides ATP for cells but also supplies ribose-5-phosphate for nucleic acid synthesis (Guo et al., 2019; Battaglia et al., 2014). Citric acid and succinic acid are key intermediates in the TCA cycle, playing critical roles in energy and substance metabolism (Zhang et al., 2020). In this study, Glucose-6-phosphate was significantly upregulated in the lysine group, suggesting that lysine may reprogram the sugar metabolism pathway in *A. parasiticus*. The accumulation of glucose-6-phosphate may reflect a slowdown in downstream metabolic pathways (e.g., glycolysis and the TCA cycle), leading to decreased energy production. Previous studies have found that fungi can redirect glucose-6-phosphate towards the pentose phosphate pathway under environmental stress, increasing NADPH production to cope with oxidative stress, but at the same time reducing energy supply and inhibiting fungal growth (Battaglia et al., 2014; Zhang et al., 2020; Poulsen et al., 2005). Niu et al. (2023) confirmed that the activation of glucose-6-phosphate dehydrogenase (a key enzyme in the pentose phosphate pathway) can significantly inhibit the mycelial growth and AFB_1 synthesis of *A. flavus*, which is consistent with the effect of lysine in this study. In addition, both citric acid and succinic acid were significantly upregulated in the lysine group in this study, further confirming that lysine can interfere with the TCA cycle function of *A. parasiticus*. The disruption of the TCA cycle in fungi significantly affects mitochondrial function, reduces ATP production, and thereby inhibits mycelial growth and spore formation (Zhang et al., 2019). Additionally, citric acid can reduce fatty acid synthesis by inhibiting acetyl-CoA carboxylase activity, thereby affecting the renewal and repair of cell membranes (Falade et al., 2018; Martins et al., 2025). Furthermore, Chen et al. (2025) found that succinate dehydrogenase inhibitors can significantly reduced fungal hyphal growth rate and spore production, and that the accumulation of succinic acid was positively correlated with the fungal growth inhibition rate, findings that are highly consistent with the effect of lysine in this study.

In recent years, research has shown that G protein-coupled receptors (GPCRs) in *Aspergillus* fungi can detect fatty acids and lipid peroxides, thereby activating the cAMP/PKA pathway to regulate fungal hyphal growth, sporulation, and metabolite synthesis (Qiu et al., 2021). In this study, both 13-L-Hydroperoxylinoleic acid and arachidonic acid were significantly downregulated in the lysine group. Decreased expression of 13-L-Hydroperoxylinoleic acid may reduce activation of the cAMP signalling pathway, thereby inhibiting spore differentiation and mycelial growth in *A. parasiticus* (Yan et al., 2017). Reduced arachidonic acid levels may decrease the structural stability of *A. parasiticus* cell membranes, thereby affecting nutrient transport and signal transduction (Ząbek et al., 2017). Previous studies have confirmed that exogenous arachidonic acid supplementation promotes mycelial growth and spore germination, whereas HPODE synthesis inhibitors can significantly reduce colony diameter and AFB_1 production in *A. flavus* (Affeldt et al., 2012). These results are highly consistent with the inhibition of AFB_1 synthesis by lysine in this study, suggesting that 13-L-HPODE and arachidonic acid, which are involved in lipid oxidation or the G protein cAMP/PKA signalling pathway may be important targets through which lysine exerts its effects.

3.7. KEGG pathway enrichment analysis of differential metabolites in *A. parasiticus* under lysine and proline stress

KEGG pathway analysis was performed on the differential metabolites of *A. parasiticus* under lysine and proline stress (Fig. 8). A total of 71 metabolic pathways were identified in the differential metabolites between the proline group and the control group (Fig. 8a), mainly including aminoacyl-tRNA biosynthesis, TCA cycle, arginine biosynthesis, ABC transporters, and oxidative phosphorylation. The pathways with higher significance included aminoacyl-tRNA synthesis and arginine biosynthesis, indicating active protein synthesis and amino acid metabolism in the samples (Pang et al., 2021). Additionally, pathways with higher Hits values (a quantitative metric assessing differential metabolite enrichment in specific pathways) included ABC transporters and cysteine/methionine metabolism, indicating a large number of differential metabolites related to transmembrane transport or redox balance regulation in these pathways (Pan et al., 2020). Furthermore, the enrichment of oxidative phosphorylation and the TCA cycle reflects significant changes in energy metabolism, which may be related to the high-energy-demand state of cells (e.g., proliferation or stress) (Vander Heiden et al., 2009). Notably, the enrichment of lysosomal degradation (Efferocytosis) and β-alanine metabolism may point to mechanisms related to cell apoptosis clearance or neural regulation (Poon et al., 2014).

A total of 70 regulated metabolic pathways were identified in the differential metabolites between the lysine group and the control group (Fig. 8b), including: 1) protein synthesis related pathways: aminoacyl-tRNA biosynthesis and amino acids biosynthesis; 2) energy metabolism pathways: TCA cycle and oxidative phosphorylation; 3) amino acid metabolic networks: including alanine/aspartate/glutamate and glycine/serine/threonine metabolism; 4) Special metabolic regulatory pathways: monomycin synthesis, riboflavin metabolism, etc. Among these, the amino acids biosynthesis pathways exhibit systematic enrichment, and the biosynthesis modules for branched-chain and aromatic amino acids are synergistically activated, suggesting that cells may support protein synthesis demands or stress adaptation through nitrogen metabolism reprogramming (Hosios and Vander Heiden, 2018). Meanwhile, mitochondrial function significantly enhances energy metabolism, and the co-enrichment of the TCA cycle and oxidative phosphorylation reflects the energy-coupling mechanism of the tricarboxylic acid electron transport chain (Martínez-Reyes and Chandel, 2020). The correlation in the D-amino acid metabolism pathway may indicate a regulatory effect of the mitochondrial/cytoplasmic α-ketoglutarate/glutamate shuttle on carbon-nitrogen balance (Tishkov and Khoronenkova, 2005). Additionally, the high impact value of the ABC transporter protein indicates that transmembrane metabolite transport plays a crucial role in maintaining system homeostasis, potentially by regulating the efflux of toxic substances or nutrient uptake (Locher, 2016).

Based on differential metabolites and their KEGG regulatory pathways of *A. parasiticus* under lysine and proline stress, we found that proline can significantly activate the energy and nitrogen metabolism network, induce the accumulation of TCA cycle intermediates (citric acid), enhance the ability of cells to synthesise ATP, and provide an energy basis for AFB_1 biosynthesis (Fig. 9). Simultaneously, 5′-deoxyadenosine and methyl gibberellin A9 were upregulated, thereby synergistically amplifying toxin production capacity by enhancing methyl donor supply and promoting hyphal elongation. In addition, the significant downregulation of lipid peroxides such as 15,16-DiHODE relieves the negative regulation of the G protein cAMP/PKA pathway, further promoting AFB_1 synthesis mediated by *aflR*. Concurrently, 5′-deoxyadenosine and methyl gibberellin A9 were upregulated, thereby synergistically amplifying toxin production by enhancing the supply of methyl donors and promoting hyphal elongation. Furthermore, the significant downregulation of lipid peroxides such as 15,16-DiHODE relieves their negative regulation of the G protein cAMP/PKA

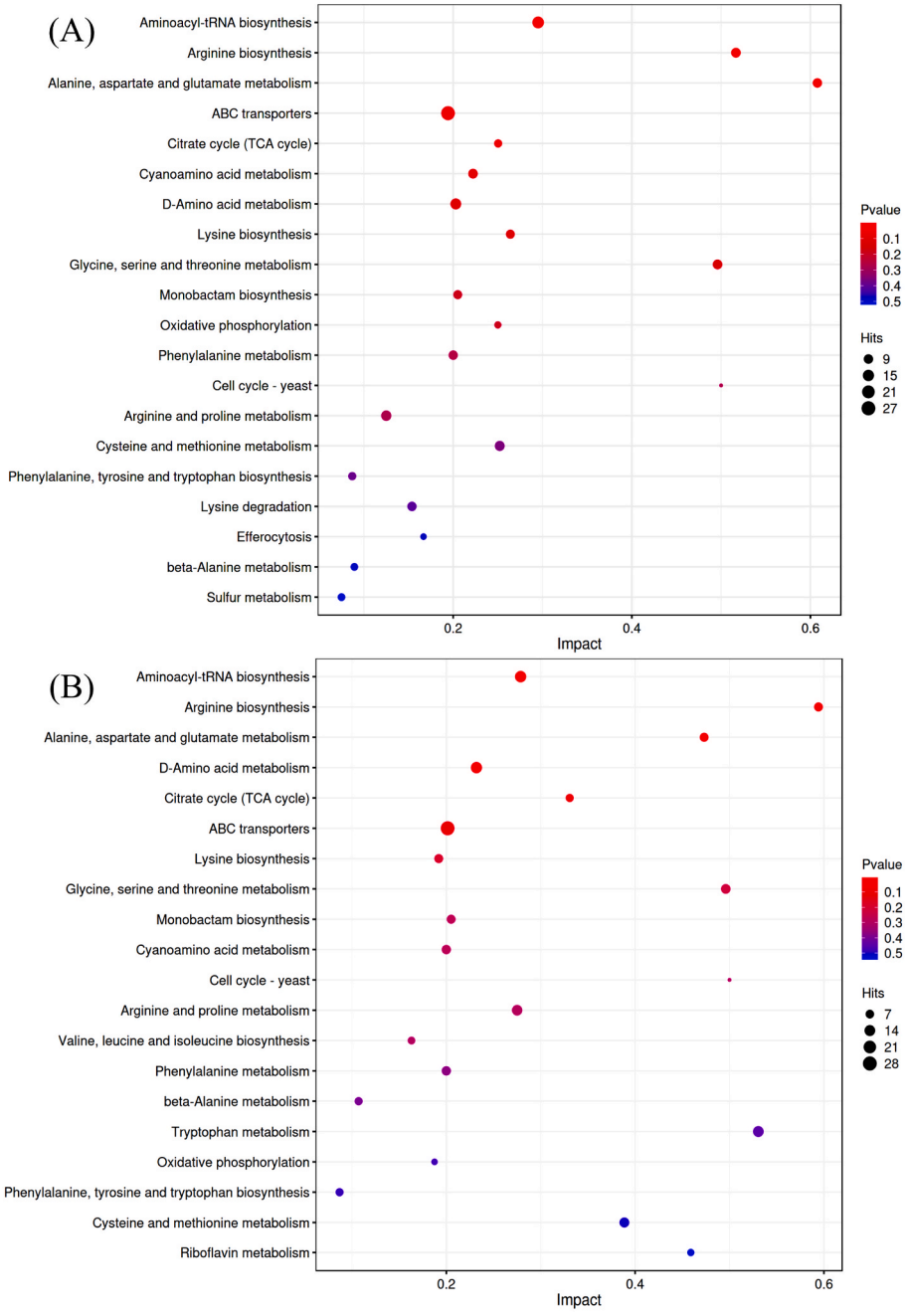


Fig. 8. KEGG pathway enrichment analysis of differentially expressed metabolites in *A. parasiticus* under proline (A) and lysine (B) stress. The x-axis represents the enrichment score, and the y-axis represents metabolic pathways. Point size indicates the number of differentially expressed metabolites (Hits), and point color represents the significance level (P value). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pathway, thereby further promoting AFB_1 synthesis. Conversely, lysine can induce a significant metabolic imbalance, leading to abnormal accumulation of glucose-6-phosphate, citric acid, and succinic acid, indicating that carbohydrate metabolism and the TCA cycle were blocked and that energy generation efficiency decreased. Concurrently, the significant downregulation of gibberellin A20 directly inhibited the mycelial growth and toxin production of *A. parasiticus*. Additionally, the levels of 13-L-Hydroperoxylinoleic acid and arachidonic acid decreased, weakening lipid signalling transduction and cell membrane integrity, and inhibiting the growth, differentiation, and toxin synthesis of *A. parasiticus* at multiple levels. Thus, the above results demonstrate that proline and lysine establish a bidirectional fine-tuning mechanism for *A. parasiticus* growth and AFB_1 biosynthesis by promoting or inhibiting energy metabolism, lipid peroxide signalling factor, and secondary metabolism pathways, respectively.

4. Conclusion

This study demonstrated that key amino acids in marinade bidirectionally regulate the growth and AFB_1 production of *A. parasiticus*. Proline, phenylalanine, and leucine promoted fungal growth and toxin synthesis, whereas lysine, arginine, and glycine exhibited inhibitory effects. Metabolomics analysis showed that proline activates the TCA cycle and the gibberellin metabolic pathway, accumulates energy metabolism intermediates, such as citric acid, and downregulates negative regulatory factors such as 15,16-DiHODE, thereby

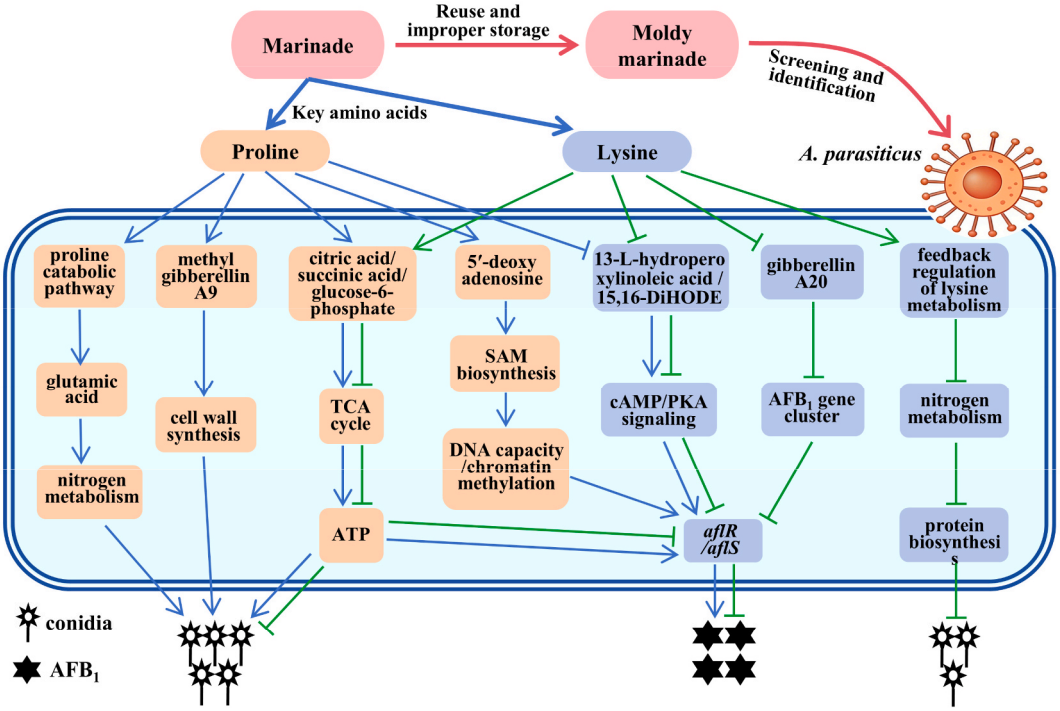


Fig. 9. Schematic diagram of the regulatory mechanisms by which proline promotes and lysine inhibits *A. parasiticus* growth and AFB_1 biosynthesis through metabolic reprogramming.

synergistically promoting *A. parasiticus* growth and AFB_1 synthesis. Lysine induces disturbances in glucose metabolism and the TCA cycle, leading to abnormal accumulation of metabolites such as glucose-6-phosphate, while downregulating key substances, including gibberellin A20, disrupting cell membrane integrity and energy supply, thereby inhibiting the growth and toxin production of *A. parasiticus* at multiple levels. This study elucidates the regulatory roles and mechanisms of key amino acids in marinade on *A. parasiticus* and AFB_1 synthesis, providing crucial technical references and a theoretical basis for preventing and controlling AFB_1 contamination in marinated product production by regulating the marinade's amino acid composition. The findings support the development of marinade formulation strategies (such as optimizing amino acid balance or incorporating lysine-enriched ingredients) to inherently suppress toxigenic fungi, thereby enhancing the safety and shelf-life of marinated foods. Combined with our previous studies on marinade microbiota and toxin screening, this work contributes to a holistic approach for managing fungal risks in traditional marinated food systems.

CRediT authorship contribution statement

Xinyue Hu: Writing – original draft, Software, Methodology, Data curation. **Xianghong Deng:** Writing – original draft, Software, Methodology, Formal analysis. **Zhanrui Huang:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Wanying He:** Visualization, Resources, Investigation. **Lianhua Hu:** Software, Methodology, Investigation. **Guoqing Wu:** Software, Methodology, Formal analysis, Data curation. **Xiaojie Zhou:** Visualization, Software, Methodology. **Hao Chen:** Resources, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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