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Original Research Article

# *Monascus purpureus* M-32 fermented soybean meal improves the growth, immunity parameters, intestinal morphology, disease resistance, intestinal microbiota and metabolome in Pacific white shrimp (*Litopenaeus vannamei*)

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ABSTRACT

This study was conducted to evaluate the effects of *Monascus purpureus* M-32 fermented soybean meal (MFSM) on growth, immunity, intestinal morphology, intestinal microbiota, and intestinal metabolome of Pacific white shrimp (*Litopenaeus vannamei*). Four groups of diets were formulated, including control group (30% fish meal and 30% soybean meal [SBM] included in the basal diet) and three experimental groups which MFSM replaced 20% (MFSM20), 40% (MFSM40), and 60% (MFSM60) of SBM in control group, respectively. Results showed that the soluble proteins larger than 49 kDa in MFSM were almost completely degraded. Meanwhile, the crude protein, acid-soluble protein, and amino acid in MFSM were increased. The results of shrimp culture experiment showed that the replacement of SBM with MFSM decreased FCR ($P < 0.001$) and content of malondialdehyde ($P = 0.007$) in the experimental groups, and increased weight gain rate ($P = 0.006$), specific growth rate ($P = 0.002$), survival rate ($P = 0.005$), intestinal villus height ($P < 0.001$), myenteric thickness ($P = 0.002$), the activities of superoxide dismutase ($P = 0.002$), and lysozyme ($P = 0.006$) in experimental groups, as well as increased content of calcium ($Ca^{2+}$) and phosphorus ($PO_4^{3-}$) in blood and muscle, and enhanced resistance to *Vibrio parahaemolyticus* infection. The gut microbiota of MFSM groups was significantly different from that of the control group, and the abundance of Actinobacteria and Verrucomicrobia increased significantly in the MFSM60 group, whereas Proteobacteria and Firmicutes decreased. Compared with the control group, there were significant changes in the levels of several intestinal metabolites in the MFSM60 group, including leukotriene C5, prostaglandin A1, taurochenodeoxycholic acid, carnosine, and itaconic acid. The fermentation of SBM by the strain *M. purpureus* M-32 has the potential to enhance the nutritional quality of SBM, promote the growth of *L. vannamei*, boost immune response, improve intestinal morphology and microbiota composition, as well as influence intestinal metabolites.


## 1. Introduction

Soybean meal (SBM), a fish meal alternative, is regarded as a preferable protein source for aquatic feed owning to its relatively high protein content, balanced amino acid profile, and steady supply (Gatlin et al., 2007). However, the application of SBM in aquatic animals is limited due to the presence of multiple anti-nutritional factors such as lectins, antigens, trypsin inhibitors, and

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oligosaccharides (Francis et al., 2001), which may have negative effects on the gut health, feed utilization, growth performance, and non-specific immune responses of aquatic animals (Liu et al., 2016; Lin and Cheng, 2017; Lin and Mui, 2017). Fermentation has been identified as an effective approach to eliminate anti-nutritional factors and convert macromolecular nutrients to small molecule nutrients (Egounlety and Aworh, 2003; Hong et al., 2004). Moreover, microbes can endow SBM with new functions by secreting functional components such as exoenzymes, vitamins, antibacterial peptides, and various short-chain fatty acids. Lactic acid bacteria-fermented SBM contains organic acid and antibacterial peptides, thus it improves palatability and inhibits pathogenic bacteria (Cui et al., 2020). *Bacillus*-fermented SBM contains various hydrolytic enzymes, such as proteases, α-amylase and β-glucosidase, which can hydrolyze macromolecules to small molecule nutrients (Dai et al., 2017). Yeast-fermented SBM contains bioactive metabolites such as β-glucan, mannan oligosaccharide, chitin, vitamins, and short peptides (Faustino et al., 2021). Filamentous fungi play an important role in fermenting feed raw materials by producing complex enzyme systems such as saccharifying enzymes, proteases, lipases, and saccharifying enzymes. These enzymes not only degrade raw materials but also increase their flavor compounds (Mukherjee et al., 2016). At present, several filamentous fungi have been used to ferment SBM, such as *Aspergillus* spp., *Mucor* spp., and *Rhizopus* spp. (Sanjukta and Rai, 2016). However, little information is available on *Monascus* spp. as a fermentation strain used to ferment SBM.

*Monascus* is a group of filamentous fungi, which can produce various secondary metabolites with various biological activities, such as antibacterial, anti-inflammatory, thus regulating the intestinal microbiota (Chen et al., 2015; Park et al., 2016). In previous studies, we isolated and identified *Monascus purpureus* M-32 (accession number CGMCC 19377) from the Chinese traditional food red mould rice, and confirmed that it was beneficial to *Litopenaeus vannamei* (Wang et al., 2021). Therefore, *M. purpureus* M-32 was chosen as the preferred strain for fermenting SBM in order to provide a functional protein material for *L. vannamei*.

Previous studies on fermented soybean meal (FSM) mainly focused on evaluating the effects on digestibility, growth performance, immunity, intestinal morphology or intestinal microbiota in some aquatic animals (Dossou et al., 2018; Liang et al., 2017; Li et al., 2020a; Lin and Chen, 2022; Refstie et al., 2005; Shao et al., 2018). However, few attempts have been made to analyze the effects of feeding FSM on intestinal metabolome. Thus, this study was conducted to develop a novel *M. purpureus* M-32 fermented soybean meal (MFSM) product and assess its efficacy in shrimp aquaculture, with particular emphasis on elucidating its effects on gut microbiota and metabolomic profiles.

## 2. Materials and methods

### 2.1. Animal ethics statement

The animal study protocol of the present study was reviewed and approved by the Ethics Committee of Animal Research Institute Committee guidelines, Jimei University, China (No. 2019-32, approval date: 5 March 2019).

### 2.2. Preparation of MFSM

The strain *M. purpureus* M-32 was incubated on potato dextrose agar medium at 30 °C for 7 d. The mature spores were washed with 2 mL sterile water and transferred to 50 mL sterile tubes. The clusters of spores were disrupted using oscillator for 30 s in order to obtain the spore suspension of *M. purpureus* M-32. The spore suspension was inoculated into liquid seed medium (3.50% rice meal, 1.20% soybean peptone, 1.20% glucose, 0.10% $KH_2PO_4$, 0.10% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0) at 1% inoculum, then the seed liquid of *M. purpureus* M-32 was obtained by shaken culture at 30 °C and 220 rpm for 3 d. The solid fermentation substrate consisted of 1 kg SBM, 12 g glucose, 1 g $KH_2PO_4$, 1 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, and 250 mL water. Then, 1 L solid fermentation substrate (5 cm thick) and 150 mL strain M-32 seed cultures were placed into 2 L sterile triangular bottles and shaken evenly. It was incubated at 30 °C for about 10 d and turned once a day to ensure even fermentation. Fermentation was terminated when the hyphae of strain M-32 grew all over the medium and turned red. Finally, the fermented SBM was dried at 60 °C, crushed, and passed through a 0.18-mm mesh screen.

### 2.3. Determination and analysis of MFSM

Crude protein was determined according to the China National Standard (GB/T 6432-2018) by using a Foss KT260 Kjeldahl nitrogen analyzer; acid-soluble protein by China Agricultural Industry Standard (NY/T 3801-2020) using a Foss KT260 Kjeldahl nitrogen analyzer; and amino acids by China National Standard (GB/T 18246-2019) using a HITACHI L-8900 amino acid analyzer. The contents of glycinin and β-conglycinin were determined by ELISA kit purchased from Beijing Longke Fangzhou Bioengineering Technology Co., Ltd (Beijing, China).

The soluble protein composition of SBM before and after fermentation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows: 0.50 g SBM and MFSM were weighed and placed in 15-mL tubes separately, then 5 mL phosphate buffer was added to dissolve the protein. After the mixture was fully shaken and mixed, it was centrifuged at 4 °C and 1600 × *g* for 10 min. The 100 μL supernatant was sucked into a 1.50-mL centrifuge tube, and then 25 μL of 5× buffer was added. Then the mixture was heated at 100 °C for 3 min and cooled to room temperature. The 8% to 16% gradient prefabricated gel purchased from Dalian Meilun Biotechnology Co. Ltd (Dalian, China) was used for electrophoretic reaction at 120 V for 40 min. It was dyed with Coomassie bright blue R-250 and finally decolorized with decolorizing solution until the bands were clear.

The contents of ergosterol were analyzed as follows: MFSM was extracted overnight with 80% ethanol solution and filtered with 0.45 μm filter membrane. The content of ergosterol in MFSM was determined by HPLC (LC 20, Shimadzu, Japan). The chromatographic conditions were as follows: C18 column, 250 mm × 4.60 mm × 5 μm; mobile phase (acetonitrile:ethanol = 60:40), flow rate 1 mL/min, detection wavelength 284 nm, and column temperature 30 °C. Standard products were purchased from Shanghai Maclin Biochemical Technology Co., Ltd (Shanghai, China).

### 2.4. Preparation of the feed and experimental conditions

Fish meal, SBM, and MFSM were used as the main sources of protein. Lecithin, fish oil, and soybean oil were used as the main sources of fat. Thirty percent fish meal and 30% SBM were included in the basal diet (control); and 20% (MFSM20), 40% (MFSM40), and 60% (MFSM60) SBM in the basal diet were replaced by MFSM in the experimental groups (Table 1). Feed ingredients were crushed and passed through a 0.25-mm mesh screen, weighed, and mixed to make a 1-mm diameter feed. Finally, the feed was naturally dried and stored at −20 °C in a refrigerator. Crude fat, moisture, and crude protein were determined according to China National Standards (GB/T 6433-2006; GB/T 6435-2014; GB/T6432-2018, respectively).

**Table 1**
Ingredients and nutrient levels of the experiment diets (%, air-dried basis).

| Item | Control | MFSM20 | MFSM40 | MFSM60 |
|---|---|---|---|---|
| Ingredients | | | | |
| Fish meal | 30.00 | 30.00 | 30.00 | 30.00 |
| SBM | 30.00 | 24.00 | 18.00 | 12.00 |
| MFSM | 0.00 | 5.21 | 10.45 | 15.70 |
| Alpha starch | 5.00 | 5.00 | 5.00 | 5.00 |
| Wheat flour | 20.60 | 21.39 | 22.15 | 22.90 |
| Shrimp meal | 5.00 | 5.00 | 5.00 | 5.00 |
| Vitamin C | 0.10 | 0.10 | 0.10 | 0.10 |
| Choline chloride | 0.50 | 0.50 | 0.50 | 0.50 |
| Sodium alginate | 1.00 | 1.00 | 1.00 | 1.00 |
| Soybean oil | 2.00 | 2.00 | 2.00 | 2.00 |
| Fish oil | 2.00 | 2.00 | 2.00 | 2.00 |
| Lecithin | 2.00 | 2.00 | 2.00 | 2.00 |
| Vitamin premix[1] | 0.40 | 0.40 | 0.40 | 0.40 |
| Mineral premix[2] | 0.40 | 0.40 | 0.40 | 0.40 |
| Monocalcium phosphate | 1.00 | 1.00 | 1.00 | 1.00 |
| Nutrient level[3] | | | | |
| Crude protein | 39.26 | 39.24 | 39.26 | 39.25 |
| Crude fat | 9.31 | 9.32 | 9.33 | 9.34 |
| Ash | 9.86 | 9.82 | 9.79 | 9.76 |
| Moisture | 10.08 | 10.01 | 9.96 | 9.88 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM.
MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively.
[1] Vitamin premix provided the following amounts per kilogram complete diet: vitamin A 8000 IU, vitamin $D_3$ 4000 IU, vitamin E 60 mg, vitamin $K_3$ 5 mg, vitamin $B_1$ 25 mg, vitamin $B_2$ 25 mg, vitamin $B_6$ 12 mg, nicotinic acid 60 mg, calcium pantothenate 50 mg, folic acid 5.3 mg, biotin 0.6 mg, vitamin $B_{12}$ 0.1 mg, inositol 150 mg.
[2] Mineral premix provided the following amounts per kilogram complete diet: $FeC_6H_5O_7$ 32 mg, $CuSO_4 \cdot 5H_2O$ 4 mg, $ZnSO_4 \cdot 7H_2O$ 30 mg, $MnSO_4 \cdot H_2O$ 12 mg, $KIO_3$ 9 mg, $Na_2SeO_3$ 4 mg, $CoCl \cdot 6H_2O$ 8 mg, $MgSO_4 \cdot H_2O$ 60 mg.
[3] Nutrient levels were measured values.

Healthy *L. vannamei* and the experiment site were provided by the Da Bei Nong Haikang breeding base (Fujian, China). The shrimps were accommodated for 1 week with salinity 32‰ to 34‰ with water temperature 30 °C. The experiment was randomly divided into four groups (Control, MFSM20, MFSM40, and MFSM60). There were 3 replicates in each group and 40 shrimp in each replicate (initial weight = 2.16 g). The shrimps were fed the control and three experimental diets (Table 1) for 6 weeks.

During the experiment, a quarter of cultivation water was renewed daily. Shrimps were fed the corresponding diet 3 times a day (08:00, 13:00, 18:00). The residual feeds were removed after 2 h of feeding. During the experiment, the health status of shrimps was observed at each meal and the death of shrimps was recorded with the dead shrimps removed timely.

### 2.5. Artificial infection experiment

A pre-experiment was performed in the early stage of the experiment, and results showed that for *L. vannamei* challenged by *Vibrio parahaemolyticus* CICC 21617, the lethal concentration of the solution was $2.50 \times 10^6$ CFU/mL. Ten shrimps were randomly selected in each experimental group, and each shrimp was injected with 15 μL suspension of CICC 21617. Death statistics were collected daily for 7 d.

### 2.6. Sample collection

After the culture experiment and fasting for 24 h, the total weight and quantity of shrimps per tank were recorded before sampling, and the feed intake of shrimps in each group was used to calculate the growth index. Hepatopancreas, hemolymph, and muscle of 5 shrimps were randomly sampled from each tank for non-specific immune response, digestive enzyme activity, calcium and phosphorus index analysis. Samples of each shrimp were quickly removed and immediately immersed in liquid nitrogen, then stored at −80 °C. The middle intestines of 5 randomly selected shrimps from each tank were stored in Bouin's solution (picric acid saturated solution:formaldehyde:ice acetic acid = 15:5:1) for tissue sections observation. The whole intestines of 6 randomly selected shrimps from each tank were sampled for intestinal microbial diversity analysis and intestinal untargeted metabolomics analysis.

### 2.7. Growth and biochemical indexes determination

Growth indexes, antioxidant and immune indexes, and intestinal sections were determined according to the methods of our previous study (Wang et al., 2021). The contents of calcium ($Ca^{2+}$) and phosphorus ($PO_4^{3-}$) in blood and muscle were determined by using the kit of Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and the experimental steps were performed in accordance with the kit instructions.

### 2.8. Intestinal microbiota analysis of L. vannamei

Total DNA was extracted by using the FastDNA Spin Kit (MP Biomedicals, USA) according to the instructions. Universal primers 338 F (5′-ACTCCGGGAGCAGCA-3′) and 806 R (5′-GGACTACH VGGTWTCTAAT-3′) were used to amplify the V3–V4 region of the 16S rRNA gene from the intestinal microbiota of *L. vannamei*. The experiment was carried out according to method of Yang et al. (2019). PCR products were purified using the AxyPrep DNA Gel Extraction Kit following the instructions. The library was constructed using the NEXTflex Rapid DNA-Seq Kit (Bio Scientific, USA). Finally, Illumina's Miseq PE300 platform was used for sequencing.

Biological information data were analyzed on the Majorbio Cloud Platform (https://cloud.majorbio.com). Quality control and preprocessing of the original sequencing data were performed using fastp software (https://github.com/OpenGene/fastp, version 0.20.0), and the reads were merged using FLASH software (http://www.cbcb.umd.edu/software/flash, version 1.2.7). Operational taxonomic units (OTU) were clustered with 97% similarity using UPARSE software (http://drive5.com/uparse/, version 7.1). Sequences were analyzed using the QIIME software (http://qiime.org/scripts/assign_taxonomy.html). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/, version 2.2) against the Silva 16S rRNA database (v138) using a confidence threshold of 70%.

The data of intestinal microbiota of *L. vannamei* have been submitted to the NCBI Sequence Read Archive (accession number PRJNA871064).

### 2.9. Intestinal metabolome analysis of L. vannamei

#### 2.9.1. Sample pretreatment

Fifty milligrams of intestinal samples were collected, and 400 μL of the extract solution (acetonitrile:methanol = 1:1) was added. After mixing for 30 s on a vibrator, ultrasonic extraction was performed at 5 °C and 40 kHz for 30 min. The samples were cooled to −20 °C and then centrifuged at 4 °C and 13,000 × *g* for 15 min. The supernatant was blown dry with nitrogen and dissolved with 120 μL solution (acetonitrile:water = 1:1). The supernatant was extracted and centrifuged again by ultrasound at low temperature, and then removed to the sample bottle for LC–MS analysis. During the instrumental analysis, one quality control (QC) sample was inserted into every 6 samples to investigate the repeatability of the whole analysis process.

#### 2.9.2. LC–MS analysis

Metabolites were chromatographic separated on a ExionLC AD system (AB Sciex, USA) equipped with an ACQUITY UPLC BEH C18 column (100 mm × 2.10 mm i.d., 1.70 μm; Waters, Milford, USA). The mobile phases consisted of 0.10% formic acid in water (0.10%) (solvent A) and 0.10% formic acid in solution (acetonitrile:isopropanol = 1:1) (solvent B). The solvent gradient are shown in Table S1. The sample injection volume was 20 μL and column temperature was 40 °C.

The UPLC system was coupled to a quadrupole-time-of-flight mass spectrometer (Triple TOF 5600+, AB Sciex, USA) equipped with electrospray ionization (ESI) source operating in positive and negative modes. The optimal conditions are shown in Table S2. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 50 to 1,000 *m*/*z*.

#### 2.9.3. Data pre-processing

The original data of LC–MS were optimized and processed by Progenesis QI (Waters Corporation, Milford, USA) to obtain the initial data matrix, which used the 80% rule to remove the missing values and normalized the peak intensity with the total-sum normalization. Variables with relative standard deviation >30% in QC samples were discarded, and the data matrix for analysis was obtained after log-transformed ($\log_{10}$). At the same time, the MS and MSMS mass spectrum information were compared with the HMDB database (http://www.hmdb.ca/) and Metlin database (https://metlin.scripps.edu/) to obtain the metabolite information. Concretely, the mass tolerance between the measured mass (*m*)-to-charge (*z*) ratios (*m*/*z*) values and the exact mass of the components of interest was ±10 ppm.

#### 2.9.4. Differential metabolite analysis

Data analysis was performed on Majorbio Cloud Platform (https://cloud.majorbio.com). Partial least squares discriminant analysis (PLS-DA) was used to evaluate the similarity of samples within the group and the difference of samples between the groups, with a 0.95 confidence level. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using Version 1.6.2, and the stability of the model was assessed using 7 cycles of interactive validation. The statistical significance was analyzed by Student's *t*-test analysis, and the metabolites with the variable importance in projection (VIP) in the OPLS-DA model (VIP_pred_OPLS-DA) > 1 and *P*-value of Student's *t*-test ($P < 0.05$) were considered to be significantly changed metabolites. The metabolic pathways enrichment of differential metabolites was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/).

#### 2.9.5. Data upload

The data of gut metabolites of *L. vannamei* have been submitted to the OMIX database of Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix) under study accession number OMIX001796.

### 2.10. Statistical analysis

SPSS 20.0 software was used to conduct one-way analysis of variance (one-way ANOVA) and Duncan multiple comparison. The data were expressed as mean ± SD, and the value of $P < 0.05$ indicated a significant difference.

## 3. Results

### 3.1. The effects of fermentation on soluble protein of SBM

The results of SDS-PAGE (Fig. S1) showed that the soluble protein profile of SBM changed greatly before and after fermentation. SBM before fermentation contains mainly macromolecular proteins with molecular weight greater than 34 kDa. The molecular weight of soluble proteins in fermented SBM were mostly less than 25 kDa. Proteins larger than 49 kDa in SBM were almost completely degraded in the fermentation process.

### 3.2. The effects of fermentation on nutrient compositions and antigenic proteins of SBM

The changes of crude protein, acid-soluble protein, amino acid composition, ergosterol, and antigenic protein contents in SBM before and after fermentation are presented in Table 2. The crude protein content of SBM increased from 46.03% to 51.60% after the fermentation, and the acid-soluble protein content increased nearly 4 times, from 5.36% to 19.16%. The contents of all kinds of amino acids increased, and the increase in glutamic acid was the highest, from 9.07% to 10.57%. The content of ergosterol, a metabolite of *M. purpureus*, reached 149.15 μg/g after fermentation.

### 3.3. Effects of replacing SBM with MFSM on growth performance of *L. vannamei*

As shown in Table 3, the weight gain rate (WGR) ($P = 0.006$) and specific growth rate (SGR) ($P = 0.002$) of *L. vannamei* in group MFSM were significantly higher than those in control group. The feed conversion ratio (FCR) in the experimental groups were significantly lower than that in the control group ($P < 0.001$), and the FCR decreased gradually with the increase of MFSM level, but there was no significant difference among the 3 experimental groups ($P = 0.207$). The survival rate (SR) in the MFSM60 group was significantly higher than that in control group ($P = 0.005$), but there was no significant difference between the MFSM60 group and the

**Table 2**
Nutritional composition and antigenic protein content of soybean meal (SBM) before and after fermentation (%, air-dried basis).

| Item | SBM | MFSM |
|---|---|---|
| Crude protein | 46.03 | 51.60 |
| Acid soluble protein | 5.36 | 19.16 |
| Lysine | 2.99 | 3.17 |
| Aspartic acid | 5.36 | 6.28 |
| Threonine | 1.81 | 2.17 |
| Serine | 2.46 | 2.86 |
| Glutamate | 9.07 | 10.57 |
| Glycine | 1.95 | 2.38 |
| Alanine | 2.07 | 2.59 |
| Valine | 2.16 | 2.67 |
| Methionine | 0.27 | 0.36 |
| Isoleucine | 2.06 | 2.50 |
| Leucine | 3.60 | 4.31 |
| Tyrosine | 1.33 | 1.64 |
| Phenylalanine | 2.31 | 2.75 |
| Histidine | 1.17 | 1.33 |
| Arginine | 3.20 | 3.55 |
| Proline | 2.35 | 2.67 |
| Glycinin, mg/g | 128.79 | 10.78 |
| β-Conglycinin, mg/g | 75.41 | 9.50 |
| Ergosterol, μg/g | 34.95 | 149.15 |

MFSM = *M. purpureus* M-32 fermented SBM.

**Table 3**
Growth performance of *L. vannamei* fed with SBM replaced by MFSM.

| Item | Control | MFSM20 | MFSM40 | MFSM60 | *P*-value |
|---|---|---|---|---|---|
| IBW, g | 2.16 ± 0.14 | 2.16 ± 0.11 | 2.16 ± 0.05 | 2.16 ± 0.11 | 0.437 |
| FBW, g | 13.43 ± 0.23 | 14.86 ± 0.86 | 15.54 ± 1.20 | 15.45 ± 0.81 | 0.054 |
| FI, g/shrimp | 15.78 ± 0.44 | 16.38 ± 0.67 | 16.19 ± 1.11 | 15.95 ± 0.36 | 0.215 |
| WGR, % | 522.32 ± 10.82$^{b}$ | 588.57 ± 39.74$^{ab}$ | 620.05 ± 55.56$^{a}$ | 615.85 ± 37.67$^{a}$ | 0.006 |
| FCR | 1.40 ± 0.01$^{a}$ | 1.29 ± 0.04$^{b}$ | 1.21 ± 0.08$^{b}$ | 1.20 ± 0.06$^{b}$ | <0.001 |
| SGR, %/d | 4.35 ± 0.0.04$^{b}$ | 4.59 ± 0.14$^{a}$ | 4.70 ± 0.18$^{a}$ | 4.68 ± 0.13$^{a}$ | 0.002 |
| SR, % | 82.50 ± 5.00$^{b}$ | 90.00 ± 6.61$^{ab}$ | 84.17 ± 10.10$^{ab}$ | 94.17 ± 1.44$^{a}$ | 0.005 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM; IBW = initial body weight; FBW = final body weight; FI = feed intake; WGR = weight gain rate; FCR = feed conversion ratio; SGR = specific growth rate; SR = survival rate.
MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively.
Data are presented as mean ± SD. $^{a, b}$Values in the same row with different superscripts indicate significant differences ($P < 0.05$).

other two experimental groups ($P = 0.289$). The SR of MFSM20 and MFSM40 groups was higher than that of control group, but there was no significant difference ($P = 0.162$).

### 3.4. Effects of replacing SBM with MFSM on the antioxidant capacity in hepatopancreas of L. vannamei

As shown in Table 4, glutathione peroxidase activity in hepatopancreas of *L. vannamei* in the experimental groups were higher than that in the control group, but there was no significant difference ($P = 0.126$). The content of malondialdehyde (MDA) in experimental group was significantly lower than that in control group ($P = 0.007$), and MFSM20 group had the lowest content. However, the MDA content was not significantly different among the 3 experimental groups ($P = 0.108$). The activity of superoxide dismutase (SOD) in experimental groups were significantly higher than that in control group ($P = 0.002$).

### 3.5. Effects of replacing SBM with MFSM on serum biochemical indexes of L. vannamei

As shown in Table 5, the activities of acid phosphatase, alkaline phosphatase and phenol oxidase in serum of *L. vannamei* in the experimental group were higher than those in the control group, but there was no significant difference. The activity of lysozyme in the MFSM60 group was significantly higher than that in control, groups MFSM20 and MFSM40 ($P = 0.006$), however, there was no significant difference between the other two experimental groups (MFSM20 and MFSM40) and the control group ($P = 0.388$).

### 3.6. Effects of replacing SBM with MFSM on calcium and phosphorus in blood and muscle of L. vannamei

As shown in Table 6, the content of $Ca^{2+}$ in muscle of *L. vannamei* in MFSM40 and the MFSM60 groups was significantly higher than that in control group and MFSM20 group ($P = 0.001$), and the difference increased significantly with the increase of substitution ratio. The content of $PO_4^{3-}$ in muscle of *L. vannamei* in the MFSM60 group was significantly higher than that in control group, MFSM20, and MFSM40 groups ($P = 0.006$). There was no significant difference in the content of $PO_4^{3-}$ ($P = 0.107$) and $Ca^{2+}$ ($P = 0.137$) in blood between the experimental group (MFSM20, MFSM40, and MFSM60) and the control group.

### 3.7. The cumulative mortality rate of L. vannamei after challenged with V. parahaemolyticus

*V. parahaemolyticus* challenge experiment was performed for 7 d, and most of the death of shrimps occurred within 1 to 3 d after challenge. As shown in Fig. 1, MFSM could significantly reduce the cumulative mortality of *L. vannamei* after the challenge of

**Table 4**
Antioxidant capacity in hepatopancreas of *L. vannamei* fed with SBM replaced by MFSM.

| Item | Control | MFSM20 | MFSM40 | MFSM60 | *P*-value |
|---|---|---|---|---|---|
| GSH-Px, U/mg protein | 38.56 ± 0.27 | 43.57 ± 3.43 | 44.99 ± 7.04 | 38.76 ± 5.21 | 0.126 |
| MDA, nmol/mg protein | 13.16 ± 2.90$^{a}$ | 5.31 ± 0.11$^{b}$ | 7.89 ± 0.53$^{b}$ | 6.53 ± 0.76$^{b}$ | 0.007 |
| SOD, U/mg protein | 6.36 ± 1.63$^{b}$ | 9.31 ± 0.51$^{a}$ | 9.64 ± 2.08$^{a}$ | 9.59 ± 1.38$^{a}$ | 0.002 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; SOD = superoxide dismutase.
MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively.
Data are presented as mean ± SD. $^{a, b}$Values in the same row with different superscripts indicate significant differences ($P < 0.05$).

**Table 5**
Serum biochemical indexes in hepatopancreas of *L. vannamei* fed with SBM replaced by MFSM.

| Indexes | Control | MFSM20 | MFSM40 | MFSM60 | *P*-value |
|---|---|---|---|---|---|
| ACP, U/L | 33.33 ± 0.15 | 39.60 ± 6.41 | 37.61 ± 11.26 | 38.02 ± 9.87 | 0.289 |
| AKP, U/L | 16.46 ± 0.53 | 17.34 ± 3.47 | 17.26 ± 2.70 | 17.14 ± 5.08 | 0.155 |
| LZM, ng/mL | 2.42 ± 0.02$^{b}$ | 2.45 ± 0.03$^{b}$ | 2.43 ± 0.01$^{b}$ | 2.69 ± 0.05$^{a}$ | 0.006 |
| PO, ng/mL | 2.44 ± 0.02 | 2.47 ± 0.03 | 2.45 ± 0.01 | 2.50 ± 0.04 | 0.077 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM; ACP = acid phosphatase; AKP = alkaline phosphatase; LZM = lysozyme; PO = phenol oxidase.
MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively.
Data are presented as mean ± SD. $^{a, b}$Values in the same row with different superscripts indicate significant differences ($P < 0.05$).

**Table 6**
Calcium and phosphorus of *L. vannamei* fed with SBM replaced by MFSM.

| Item | Control | MFSM20 | MFSM40 | MFSM60 | *P*-value |
|---|---|---|---|---|---|
| Blood | | | | | |
| $Ca^{2+}$, mmol/L | 1.98 ± 0.02 | 2.08 ± 0.03 | 2.03 ± 0.05 | 2.21 ± 0.27 | 0.137 |
| $PO_4^{3-}$, mmol/L | 1.39 ± 0.17 | 1.45 ± 0.09 | 1.22 ± 0.09 | 1.34 ± 0.45 | 0.107 |
| Muscle | | | | | |
| $Ca^{2+}$, mmol/g protein | 0.10 ± 0.01$^{c}$ | 0.10 ± 0.01$^{c}$ | 0.22 ± 0.04$^{b}$ | 0.31 ± 0.06$^{a}$ | 0.001 |
| $PO_4^{3-}$, mmol/g protein | 0.46 ± 0.01$^{b}$ | 0.42 ± 0.09$^{b}$ | 0.45 ± 0.19$^{b}$ | 1.06 ± 0.42$^{a}$ | 0.006 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM.
MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively.
Data are presented as mean ± SD. $^{a, b}$Values in the same row with different superscripts indicate significant differences ($P < 0.05$).

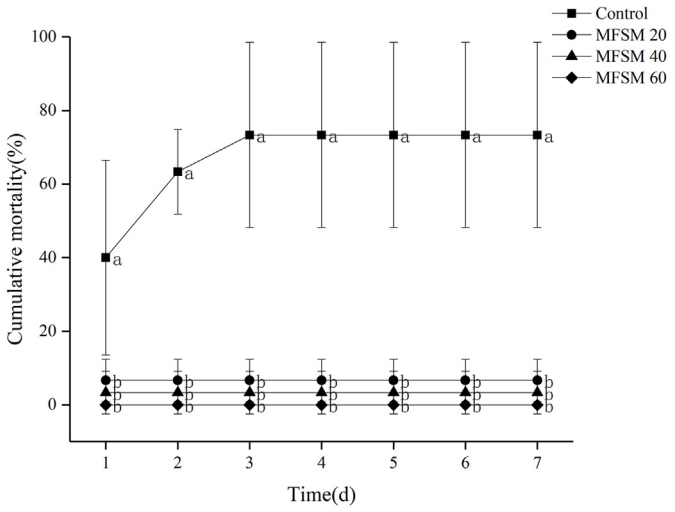


**Fig. 1.** The cumulative mortality of *L. vannamei* after challenged with *V. parahaemolyticus*. MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively. SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM. Data are presented as mean ± SD. $^{a, b}$Values with different superscripts indicate significant differences ($P < 0.05$).

*V. parahaemolyticus*. In the control group, the mortality rate in the first day was 40%, and the cumulative mortality rate in the third day reached 73.30%, and then plateaued. The death of *L. vannamei* in groups MFSM20 and MFSM40 mainly occurred on the first day, and the cumulative mortality rates were 6.67% and 3.33%, respectively, whereas the cumulative mortality rate of shrimps fed with MFSM60 was 0%.

### 3.8. Effects of replacing SBM with MFSM on the intestinal morphology of L. vannamei

The intestinal morphology of *L. vannamei* after feeding with MFSM is shown in Fig. 2. In the control group, the myenteron was thinner and the intestinal epithelial cells were separated from the basement membrane (as shown by the X arrow in Fig. 2A). The intestinal epithelial cells of the MFSM20 group were slightly separated from the basement membrane, and the intestinal epithelial cells of the MFSM40 and the MFSM60 groups were closely connected with the basement membrane. With the increase of replacement ratio, the intestinal villus height (VH) of the *L. vannamei* was significantly increased ($P < 0.001$). The VH of *L. vannamei* in MFSM40 and the MFSM60 groups were significantly higher than that in control group. The myenteric thickness (MT) in MFSM20, MFSM40, and the MFSM60 groups were significantly higher than that in control group ($P = 0.002$).

### 3.9. Effects of replacing SBM with MFSM on the intestinal microbiota of L. vannamei

The SBM in the basal diet was replaced by MFSM with different proportions. The immune indexes, intestinal tissue structure, calcium and phosphorus metabolism, survival rate after culture, and survival rate after challenge of *L. vannamei* were better in MFSM groups than those in the control group. In summary, the indexes of the MFSM60 group were more advantageous than those of other groups, especially in the survival rate after culture and survival rate after *V. parahaemolyticus* challenge. Therefore, the control and the MFSM60 groups were selected for intestinal microbiota and metabolomics analysis.

The alpha diversity statistics of intestinal microbiota of *L. vannamei* are shown in Table 7, the Sobs ($P = 0.002$), Chao ($P = 0.005$) and ACE ($P = 0.014$) indexes of group MFSM60 were significantly lower than those of control group, and there were no significant differences in Shannon ($P = 0.063$) and Simpson ($P = 0.327$) indexes among different groups ($P > 0.05$). The results showed that adding MFSM significantly reduced the richness of intestinal microbiota, but had no apparent effects on the diversity of intestinal microbiota.

At the phylum level, Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Firmicutes were the most important phyla in the intestine of shrimps (Fig. 3A). The relative abundance of major microbial phyla varied between different groups. The abundance of Actinobacteria and Verrucomicrobia increased significantly in the MFSM60 group, whereas Proteobacteria and Firmicutes decreased in the MFSM60 group (Table S3). At the genus level, there were 15 genera with relative abundance greater than 2%: *unclassified_f_Flavobacteriaceae*, *unclassified_f_Rhodobacteriaceae*, *Ruegeria_Roseobacteraceae*, *Demequina_ Demequinaceae*, *Vibrio_Vibrionaceae*, *Pseudoruegeria_Roseobacteraceae*, *Shewanella_Shewanellaceae*, *Spongiimonas_Weeksellaceae*, *Hoppeia_Flavobacteriaceae*, *Haloferula_Verrucomicrobiaceae*, *Shimia_Roseobacteraceae*, *Lactobacillus_Lactobacillaceae*, *Rhodococcus_Nocardiaceae*, norank_f_Flavobacteriaceae, and norank_f_Propionibacteriaceae (Fig. 3B). Genera *Demequina_Demequinaceae*, *Vibrio_Vibrionaceae*, *Pseudoruegeria_Roseobacteraceae*, *Hoppeia_Flavobacteriaceae* and *Haloferula_Verrucomicrobiaceae* were increased in the experimental group, whereas *Ruegeria_Roseobacteraceae*, *Shewanella_Shewanellaceae*, *Spongiimonas_Weeksellaceae*, *Shimia_Roseobacteraceae*, *Lactobacillus_Lactobacillaceae* and *Rhodococcus_Nocardiaceae* were decreased in the experimental group (Table S4).

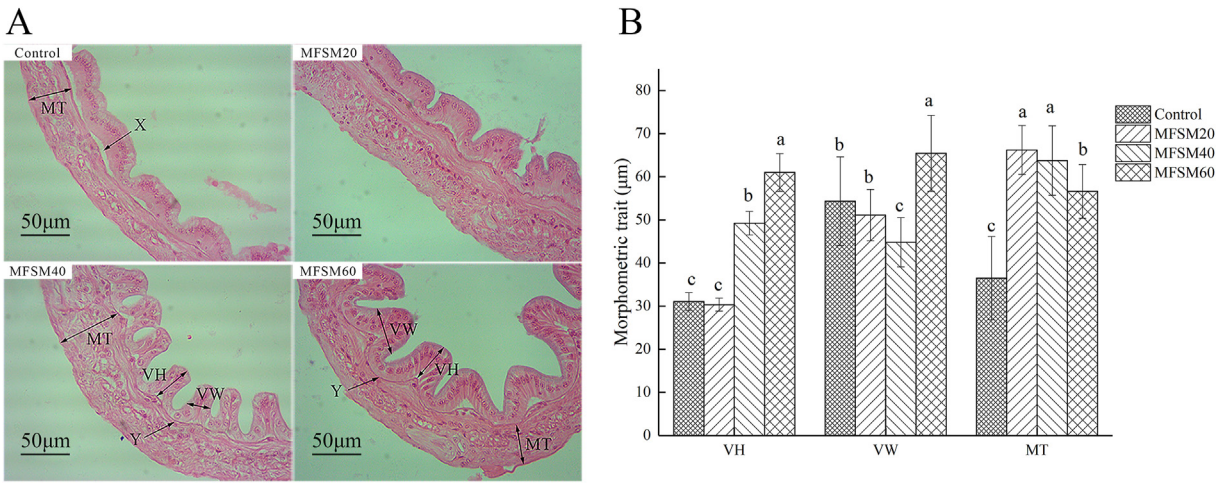


**Fig. 2.** Effects of replacing soybean meal with MFSM on the intestinal morphology of *L. vannamei*. MFSM20, MFSM40 and MFSM60 mean that 20%, 40% and 60% SBM in the basal diet were replaced by MFSM, respectively. (A) Photomicrographs (magnification 400×). Arrow X means that the intestinal epithelial cell was separated from the basement membrane; arrow Y means that the intestinal epithelial cells were closely connected with the basement membrane. (B) Morphometric trait. [a–c]Bars with different superscripts represent significant differences ($P < 0.05$). SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM; MT = myenteron thickness; VH = villus height; VW = villi width.

**Table 7**
Alpha diversity of *L. vannamei* intestinal microbiota after adding MFSM to feed.

| Item | Control | MFSM60 | *P*-value |
|---|---|---|---|
| Sobs | 344 ± 112[a] | 172 ± 24[b] | 0.002 |
| Shannon | 3.01 ± 0.43 | 2.74 ± 0.18 | 0.063 |
| Simpson | 0.12 ± 0.08 | 0.12 ± 0.02 | 0.327 |
| ACE | 402.66 ± 137.95[a] | 264.36 ± 48.25[b] | 0.014 |
| Chao | 394.37 ± 132.31[a] | 232.77 ± 33.70[b] | 0.005 |

SBM = soybean meal; MFSM = *M. purpureus* M-32 fermented SBM.
MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group.
Data are presented as mean ± SD. [a, b]Values in the same row with different superscripts indicate significant differences ($P < 0.05$).

PCoA (Fig. 4) and NMDS (Fig. S2) results showed that the intestinal microbial community of the MFSM60 group was separated from the control group and had obvious clustering, indicating that MFSM had a significant effect on the intestinal microbial of *L. vannamei*.

### 3.10. Effects of replacing SBM with MFSM on the intestinal metabolome of L. vannamei

Partial least squares discriminant analysis and OPLS-DA were performed on samples in the MFSM60 group and control group to evaluate the metabolites differences between the samples,

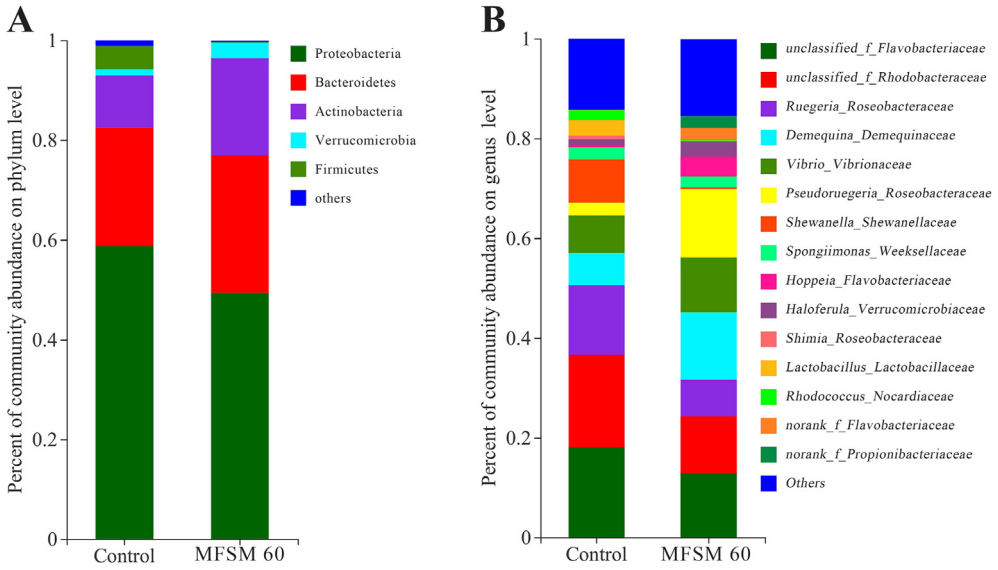


**Fig. 3.** The microbial composition of *L. vannamei* at (A) phylum and (B) genus level. MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group. SBM = fermented soybean meal; MFSM = *Monascus purpureus* M-32 fermented SBM. Phyla with relative abundance <1% were merged into others; genera with relative abundance <2% were merged into others.

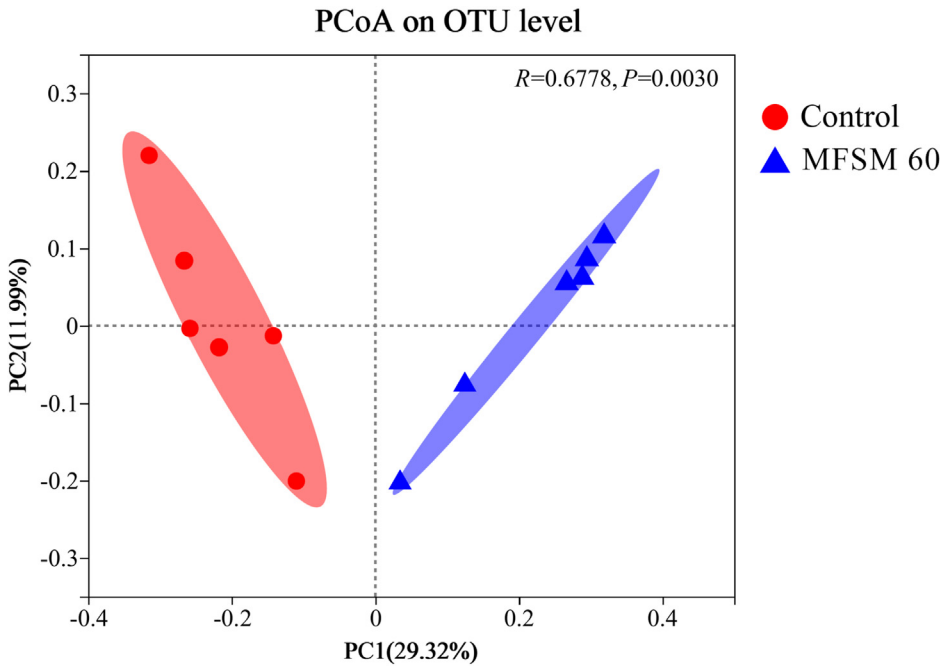


**Fig. 4.** PCoA analysis of intestinal microbiota of *L. vannamei*. MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group. SBM = fermented soybean meal; MFSM = *Monascus purpureus* M-32 fermented SBM.

respectively. As shown in Fig. S3 and Fig. 5, samples from the control group and MFSM60 group were clustered separately under positive and negative modes, indicating that MFSM had a significant impact on intestinal metabolism of *L. vannamei*. The clustered QC samples of PLS-DA confirmed the repeatability and stability of the instrument and the reliability of the data. PLS-DA and OPLS-DA model validation diagram showed that Y-axis intercepts of Q2 were both below 0 under positive and negative ion

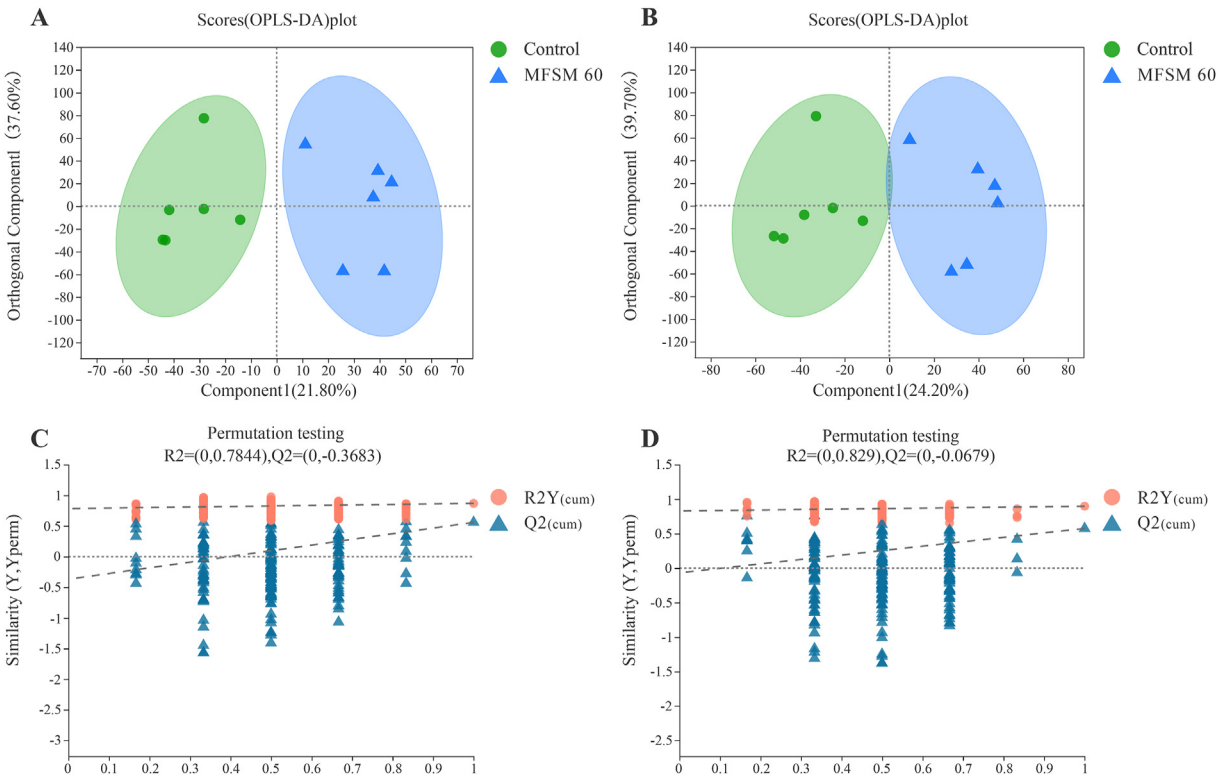


**Fig. 5.** Analysis of (A, B) orthogonal partial least squares discriminant (OPLS-DA) plot and (C, D) permutation testing of intestinal metabolites in comparisons of the Control and MSFM60 groups following (A, C) positive and (B, D) negative mode ionization. R2Y (cum) and Q2 (cum) indicate the cumulative interpretation power and predictive power of the model, respectively. MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group. SBM = fermented soybean meal; MFSM = *Monascus purpureus* M-32 fermented SBM.

modes, which indicated that the model was not overfitted and reliable.

To screen the differential metabolites, the VIP_pred_OPLS-DA > 1 and $P < 0.05$ were used as the criteria. The results showed that 4796 different ion peaks were detected between the MFSM60 group and the control group, and 139 annotated metabolites (62 and 77 metabolites in the positive and negative ion modes, respectively) were identified, of which 54 metabolites were up-regulated and 85 were down-regulated. Furthermore, 0.80 < fold change (FC) < 1.20 was used as a screening condition to screen out metabolites with higher fold-changes. As shown in Table S5, a total of 19 differential metabolites were screened, of which 10 metabolites were up-regulated and 9 were down-regulated.

KEGG pathway function and enrichment analysis showed that a total of 33 differential metabolites were annotated to the KEGG pathway, which were annotated into 5 major categories: metabolism (17), organismal systems (9), human diseases (3), environmental information processing (2), and drug development (2) (Fig. 6A). The results of KEGG pathway enrichment analysis are shown in Fig. 6B. Compared with the control group, the PPAR signaling pathway, progesterone, androgen and estrogen receptor agonists/antagonists, glycosaminoglycan degradation, eicosanoids, cholesterol metabolism, arachidonic acid metabolism were significantly affected in the MFSM60 group ($P < 0.05$). Steroid hormone biosynthesis, bile secretion, serotonergic synapse, neuroactive ligand–receptor interaction and malaria were extremely significantly affected ($P < 0.01$). The difference metabolites annotated in KEGG functional pathway were compared between groups. It was found that the contents of leukotriene C5 ($P = 0.037$) and prostaglandin A1 (PGA1) ($P = 0.042$) in the MFSM60 group were significantly higher than those of control group, whereas the contents of taurochenodeoxycholic acid (TCDCA) ($P = 0.003$), carnosine ($P = 0.006$) and itaconic acid ($P = 0.045$) were significantly lower than those of control group (Fig. 7).

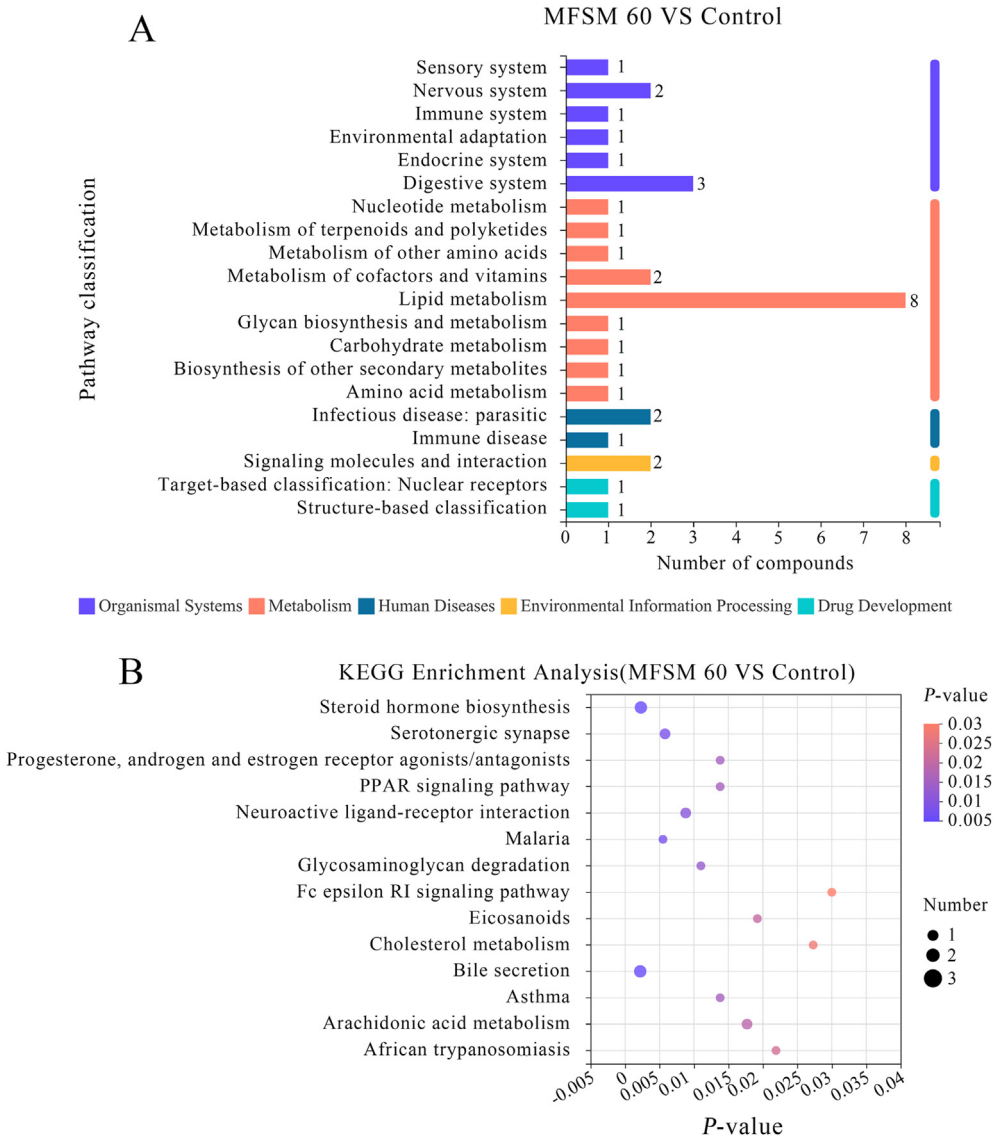


**Fig. 6.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway function and enrichment analysis and the results of KEGG pathway enrichment analysis. (A) KEGG pathways classification of differential metabolites. (B) KEGG pathway enrichment of differential metabolites. MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group. SBM = fermented soybean meal; MFSM = *Monascus purpureus* M-32 fermented SBM.

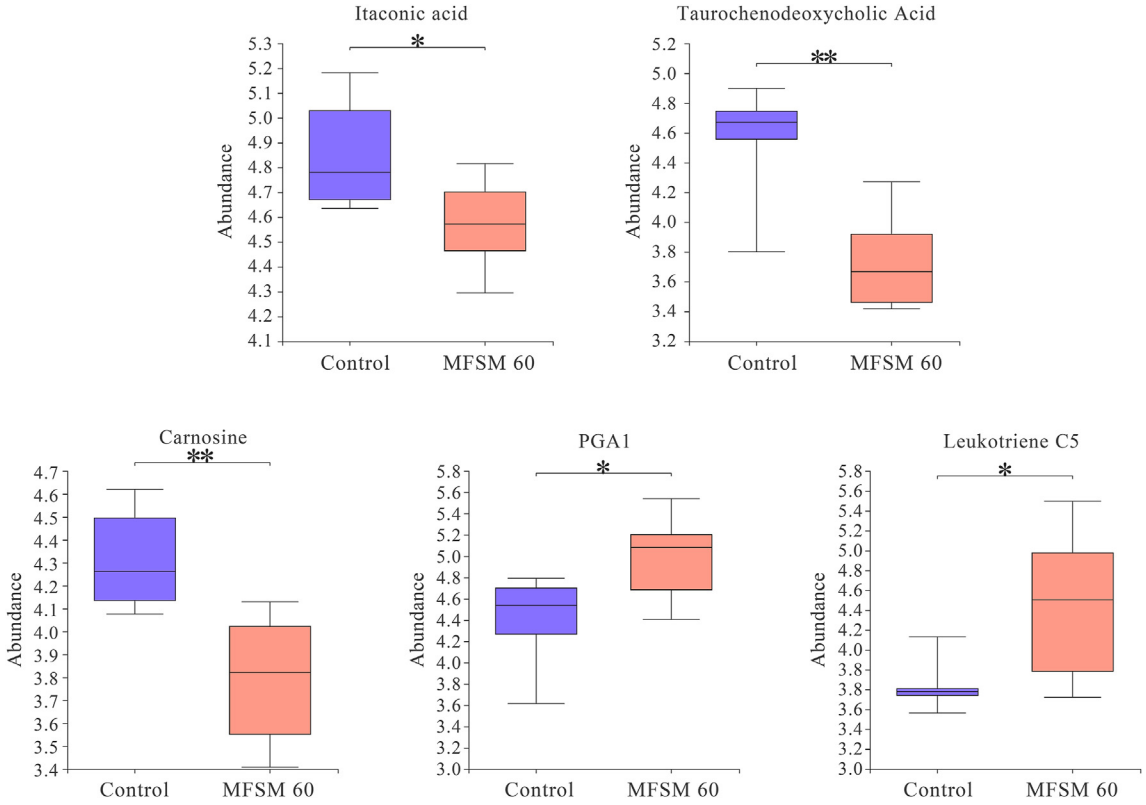


**Fig. 7.** Relative abundances of functional differential metabolites of *L. vannamei*. MFSM60 means that 60% SBM in the basal diet were replaced by MFSM in the experimental group. MFSM = *Monascus purpureus* M-32 fermented soybean meal. SBM = fermented soybean meal. *Significant difference ($P < 0.05$); **highly significant difference ($P < 0.01$).

### 3.11. Correlation analysis of intestinal microbiota with the metabolome of L. vannamei

At the genus level, Pearson correlation analysis was used to analyze the association between the top 50 intestinal microorganisms with the top 50 differential metabolites. As shown in Fig. 8, *Acinetobacter* has a significant negative correlation with 18 different metabolites such as 2-ethyl-2-hydroxybutyric acid ($P = 0.025$), 3β-hydroxy-23,24-bisnorchol-5-enic acid ($P = 0.042$), and deoxyadenosine monophosphate ($P = 0.036$). However, it was significantly positive correlated with 18 different metabolites, such as gamma-glutamylphenylalanine ($P = 0.026$) and 2-hydroxy-2-methylpropanoic acid ($P = 0.006$). *Maribacter* was positively correlated with 9 different metabolites such as adenosine monophosphate ($P = 0.033$), whereas it was significantly negative correlated with 8 differential metabolites such as 2-hydroxy-2-methylpropanoic acid ($P = 0.041$). Moreover, *Maribacter*, *Pseudahrensia*, *Pseudoalteromonas* and *Demequina* had similar correlation with differential metabolites. *Acinetobacter*, *Ralstonia*, *Rhodococcus*, *Ruegeria*, *Bacillus*, *Tenacibaculum*, and *Filomicrobium* showed similar trends in the correlation with differential metabolites.

## 4. Discussion

### 4.1. Effects of M. purpureus fermentation on the quality of SBM

Microbial fermentation is an effective method to degrade anti-nutritional factors in SBM (Lai et al., 2019). Previous studies have clearly shown that fermented SBM with probiotics can increase the contents of crude protein, crude fat and small peptides in SBM, and can also reduce the contents of glycinin, β-conglycinin and aflatoxin (Guo et al., 2022; Li et al., 2021). Some previous studies have suggested that the contents of small peptides and acid soluble protein in soybean were increased and the functional properties of proteins were enhanced after *Monascus* fermentation (Liu et al., 2009; Lim et al., 2010). *M. purpureus* M-32 was used to ferment SBM in this study, and the contents of crude protein, acid-soluble protein, and various amino acids in SBM after fermentation were significantly increased. SDS-PAGE analysis showed that there were mainly lower molecular weight proteins after SBM fermentation, and the original macromolecular proteins larger than 49 kDa were almost completely degraded. Interestingly, an increased level of ergosterol, one of the metabolites of *M. purpureus* M-32, was also detected in SBM. Results indicated that the fermentation by *M. purpureus* M-32 could improve the quality of SBM significantly, so it is feasible to prepare functional feeds by microbial fermentation. Therefore, animal experiments were used for further efficacy evaluation.

### 4.2. Effects of replacing SBM with MFSM on growth performance of L. vannamei

In this study, MFSM was used to replace SBM in the basic diet. Results showed that with the increase of replacement ratio, the FCR decreased gradually, but the specific growth rate, weight gain rate, and survival rate increased significantly. The reasons for this may be that the increase of protein digestibility is beneficial to the absorption and utilization of *L. vannamei*. On the other hand, the damage of anti-nutrient factors in SBM to *L. vannamei* was reduced through fermentation. In addition, functional components such as ergosterol and amino acids produced by *M. purpureus* M-32 may also exerted a promotion effect. The histopathological examination of the intestinal sections also revealed that MFSM promotes the health of *L. vannamei* and gradually improved the growth

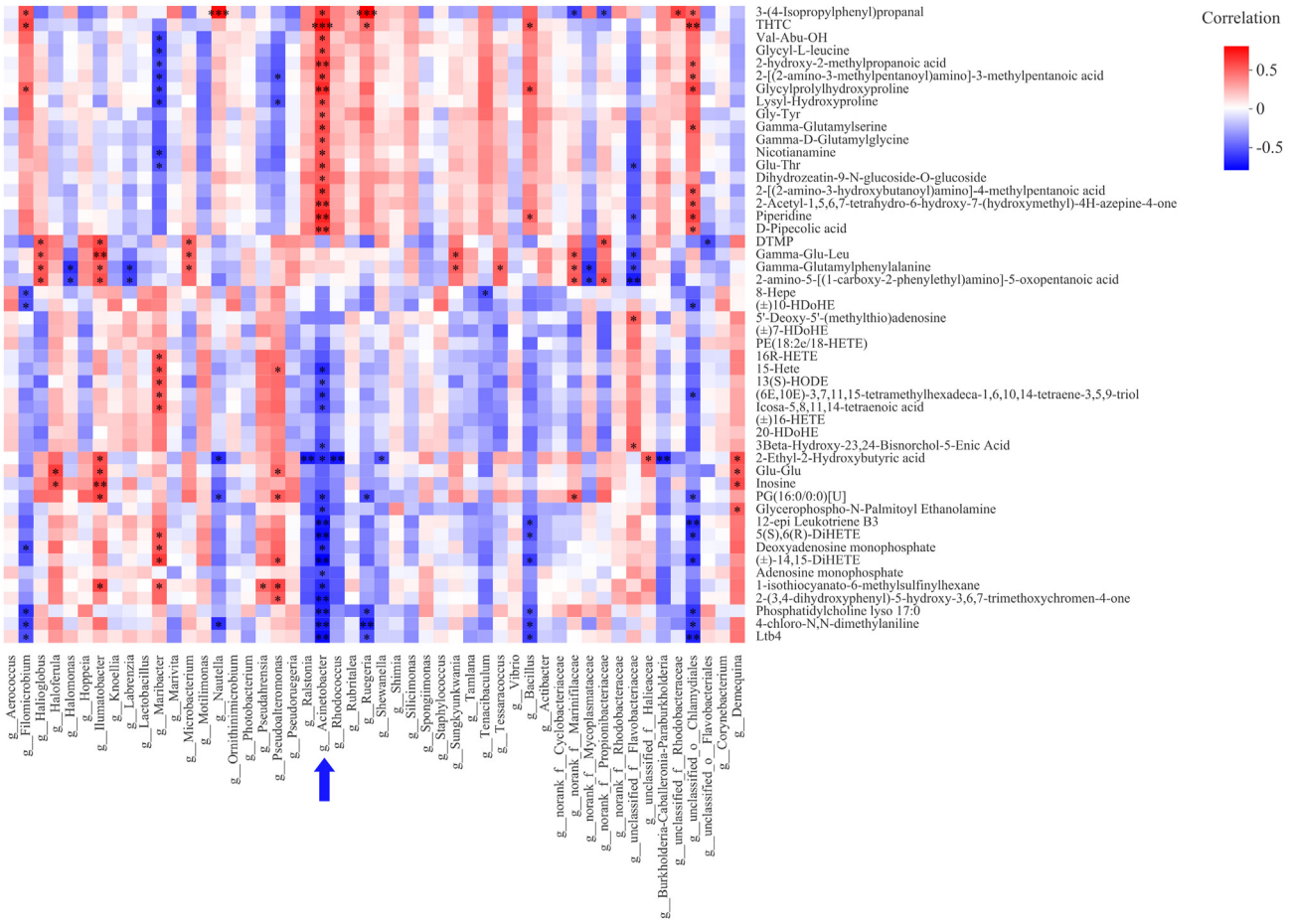


**Fig. 8.** The heatmap of correlation between intestinal microbiota and differential metabolites. The abscissa represents intestinal microbes and the ordinate represents metabolites. Each box in the figure represents the correlation between the two. Red color represents a positive correlation and blue color represents a negative correlation. *Significant difference ($P < 0.05$); **highly significant difference ($P < 0.01$).

performance of *L. vannamei*. Li et al. (2022) suggested that compared with nontreated group, *Lactobacillus* and *Saccharomyces* fermented SBM remarkably improved the weight gain rate and specific growth rate of Nile tilapia (*Oreochromis niloticus*). However, the results of Li (2020) showed that the specific growth rate, weight gain rate, and survival rate of *Anguilla japonica* decreased when fermented SBM was substituted for all SBM in the diet. According to studies by Li et al. (2015), replacing 50% or 100% SBM in feed with fermented SBM had no significant effect on specific growth rate and FCR of *Cyprinus carpio*. This may be related to the selected strains, the sensitivity of cultured animal, and the replacement ratio.

### 4.3. Effects of replacing SBM with MFSM on immune function and disease resistance of L. vannamei

In this study, the fermented SBM by *M. purpureus* M-32 could significantly reduce the cumulative mortality of *L. vannamei* after *V. parahaemolyticus* challenge. The results of immune index determination showed that SOD activity of *L. vannamei* was increased and MDA content was decreased by the fermentation of SBM by *M. purpureus* M-32. The activity of glutathione peroxidase, acid phosphatase, alkaline phosphatase, phenol oxidase, and lysozyme also increased but the difference was not significant. Previous studies have shown that soybean peptide have antioxidant and immune-enhancing functions (Chatterjee et al., 2018). In this study, large molecular weight proteins were hydrolyzed into small molecular proteins by fermentation, which may have promoted the improvement of antioxidant immune indexes and disease resistance of *L. vannamei*. In addition, the mycelium and fermentation products of *M. purpureus* M-32 were contained in fermentation substrate, which may improve the disease resistance of *L. vannamei*. *Monascus* are able to produce various secondary metabolites, including ergosterol, pigments, monacolin K, antimicrobial substances, and a variety of digestive enzymes, which exhibit a broad range of biological functions, such as regulating the intestinal microbiota, improving digestion, and they also showed antimicrobial, antimutagenic, cholesterol-lowering, and anti-inflammatory activities (Adin et al., 2023; Chen et al., 2015; Patakova, 2013). However, it will be worthwhile to further investigate which specific component plays the dominant role.

### 4.4. Effects of replacing SBM with MFSM on calcium and phosphorus in blood and muscle of L. vannamei

The amount of calcium ($Ca^{2+}$) and phosphorus ($PO_4^{3-}$) in muscle of *M. purpureus* M-32 increased significantly as the proportion of MFSM replacing SBM increased. Calcium and phosphorus not only are components of the organism but also participate in a variety of physiological metabolism as well as play a great role in life activities. The lack of calcium and phosphorus can inhibit the growth of shrimps (An et al., 2018), and the absorption and accumulation of calcium ($Ca^{2+}$) and inorganic phosphorus (Pi) are affected by

vitamin D (Zhang, 2006). Ergosterol is an important steroid compound, which exists in a variety of fungi. Yong et al. (2020) found that ergosterol from *Monascus anka* have the ability to protect and repair damaged cells. In addition to this, ergosterol is a precursor of vitamin D and can be converted into vitamin D under light conditions, so it has a positive effect on preventing osteoporosis and promoting the absorption of calcium and phosphorus (Fan, 2013; He, 2013). In this study, the concentration of ergosterol reached 149.15 μg/g after the fermentation of SBM by *M. purpureus* M-32, which may be the reason for the increase of $Ca^{2+}$ content in *L. vannamei*. However, this hypothesis still needs further exploration.

### 4.5. Effects of replacing SBM with MFSM on the intestinal microbiota of L. vannamei

Intestinal microbiota plays a pivotal role in the growth and health of aquatic animals, which are affected by many factors such as feed, water environment, animal species, and development stage. Meanwhile, intestinal microbiota can also affect the feed digestion and absorption, immune function, growth, and development of aquatic animals. Recent studies have found that fermented SBM can change the structure and relative abundance of intestinal microorganisms of aquatic animals (Catalán et al., 2018; Wang et al., 2019). In this study, MFSM significantly reduced the Chao and ACE indexes of intestinal microbiota of *L. vannamei*, but had no significant effect on Shannon and Simpson indexes, which was similar to the results of Yang et al. (2022) on *Micropterus salmoides*. However, according to the research of Shao et al. (2019), replacing fish meal with SBM fermented by complex bacteria (*Saccharomyces cerevisiae*, *Bacillus subtilis* and *Bifidobacterium*) in shrimp diet had no effect on the diversity of intestinal microbiota. The research of Li et al. (2020b) showed that feeding *Scophthalmus maximus* with fish meal instead of *Streptococcus faecium* fermented SBM could significantly increase the Chao and ACE indexes of intestinal microbiota. These results indicated that the effects of fermented SBM on intestinal microbiota of aquatic animals are not only related to the nutritional composition of SBM itself, but also related to the fermentation strains and aquatic animal species, and the detailed mechanism needs to be investigated profoundly in the future.

The present study found that Proteobacteria was absolutely dominant in all groups, followed by Bacteroidetes and Actinobacteria. The abundance of these three phyla accounted for more than 90%, among which Proteobacteria was the predominant flora for decomposing and utilizing protein sources (Wang et al., 2016). Some strains of Verrucomicrobia have the function of utilizing complex carbohydrates (Feng, 2021). Studies have shown that a large number of Verrucomicrobia colonize in the intestinal tract after treatment with broad-spectrum antibiotics, but there is no large-scale disturbance of intestinal functions (Dubourg et al., 2013), indicating that Verrucomicrobia may play an important role in stabilizing the microecological balance in the intestine. Results showed that the abundance of Verrucomicrobia increased significantly in the MFSM60 group, which could increase the stability of intestinal microecology and then positively affect the intestinal structure and survival rate of *L. vannamei*. At the genus level, the abundance of *Vibrio* in the MFSM60 group was increased, but shrimps did not show related symptoms, which was speculated to be due to the increased abundance of non-pathogenic *Vibrio*. This study also found that the abundance of *Pseudogergiella* and *Demequina* increased significantly in experimental groups. The functions of these two bacteria in intestinal microecology of shrimps are still unclear and requires further exploration.

### 4.6. Effects of replacing SBM with MFSM on the metabolome of L. vannamei

In this study, non-targeted metabolomics were used to reveal the changes in intestinal metabolome of *L. vannamei* after replacing SBM with MFSM in the basal feed. PLS-DA analysis showed that intestinal metabolites in experimental groups had significant changes. Among them, more differential metabolites were annotated to the lipid metabolism pathway in the KEGG database, suggesting that fermented SBM by *Monascus* had a greater effect on intestinal lipid metabolism of *L. vannamei*. KEGG pathway enrichment analysis showed that differential metabolites were mainly enriched in the PPAR signaling pathway, progesterone, glycosaminoglycan degradation, eicosanoids, cholesterol metabolism, arachidonic acid metabolism, steroid hormone biosynthesis pathway, synaptic serotonin pathway.

Leukotriene C5, a derivative of eicosapentaenoic acid, has a similar function as Leukotriene C4 (Hammarström, 1980). It can be used as an inflammatory mediator, interacting with a variety of cytokines and inflammatory mediators. Prostaglandins (PGs) are a class of biologically active hormone compounds produced and released by the organism under physiological or pathological stimuli, which have the function of maintaining homeostasis in the body (Naruniya, 2007), and they often act by participating in the mediation of other autologous active substances, neurotransmitters, and hormones (Gao et al., 2007). In this study, the contents of Leukotriene C5 and Prostaglandin A1 of *L. vannamei* in experimental group were up-regulated, indicating that *Monascus* fermented SBM has a stimulating effect on the inflammatory regulatory network, and further investigation is needed to unveil the detailed mechanism.

Taurochenodeoxycholic acid (TCDCA), a kind of conjugated bile acids, has an inhibitory effect on inflammatory reactions caused by physical, chemical, and bacterial factors (Li et al., 2008). Further studies have found that TCDCA can significantly improve the activities of SOD and CAT in mouse plasma or liver tissue, indicating that TCDCA has an antioxidant function (Yang and Li, 2006). Targets such as mTOR and PPAR may play an important role as core nodes in the antioxidant function of TCDCA (Liu et al., 2020). In this study, it was found that TCDCA was significantly down-regulated in experimental group. Meanwhile, KEGG pathway enrichment analysis found that the PPAR pathway was also significantly affected. It is therefore reasonable to speculate that *Monascus* fermented SBM could affect the anti-inflammatory antioxidant network through affecting the metabolism of TCDCA.

Carnosine, an important antioxidant and free radical scavenger, interacts with free radicals in cells to regulate the level of reactive oxygen species (Decker et al., 2001; Pavlov et al., 1993). There are few studies on the application of carnosine in aquatic feed and the additive amount and effect are not consistent. Previous research found that the growth performance of *S. maximus* was not affected by carnosine (supplemental level $\leq$ 100 mg/kg), but significantly decreased when supplemental level was 200 mg/kg (Wang et al., 2017). In this study, the carnosine content was significantly down-regulated after SBM was substituted by MFSM. These results suggested that MFSM might affect the antioxidant effect of *L. vannamei* by regulating metabolism of carnosine. However, there are few studies on the application of carnosine in aquatic products, so further studies are needed.

Itaconic acid is an immunomodulatory derivative derived from the tricarboxylic acid cycle, which has antibacterial, antiviral, antioxidant, anti-inflammatory, and nutritional metabolic regulation functions (Wu et al., 2021; Zhu, 2020). In this study, it was

found that the content of itaconic acid in the experimental group was significantly down-regulated. These results showed that MFSM may affect the immune regulation of *L. vannamei* by affecting the metabolism of itaconic acid.

### 4.7. Correlation analysis of intestinal microbiota with the metabolome of L. vannamei

The combined analysis of metabolomics and microbial diversity can be used to analyze the underlying mechanism of the interaction between intestinal microbes and metabolites. Liu (2019) found that the elevation of Proteobacteria and Clostridium might affect the function of vitamin E and thus induce cystitis through the combined analysis of 16S rDNA sequencing and metabolome. In this study, *Acinetobacter* was found to have the strongest correlation with differential metabolites, showing a significant negative correlation with 18 different metabolites and also a significant positive correlation with 18 different metabolites, indicating that *Acinetobacter* has a strong correlation with intestinal differential metabolites of shrimp. Huang et al. (2020) found that *Acinetobacter venetianus* is the potential pathogen of red leg disease of white leg shrimp. Beyond this, the genus *Acinetobacter* is a potential pathogen in aquaculture which has caused a large number of deaths in Indian major carp, common carp, blunt snout bream, channel catfish and silver carp in recent years (Behera et al., 2017; Cao et al., 2016; Kozinska et al., 2014; Malick et al., 2020). The study on intestinal microbial diversity in this study also found that *Acinetobacter* is one of the major difference species, and its abundance significantly decreased in MFSM groups. Thus, it is reasonable to speculate that *Monascus* fermentation of SBM may affect the intestinal metabolism of *L. vannamei* by affecting the abundance of *Acinetobacter*, and the genus *Acinetobacter* is closely related to the health of *L. vannamei*.

## 5. Conclusion

After fermentation by *M. purpureus* M-32, the contents of crude protein and acid-soluble protein in SBM were increased, with the ergosterol content reaching 149.15 μg/g. Meanwhile, the macromolecular proteins were degraded into proteins with a molecular weight less than 25 kDa. In this study, MFSM was used to replace SBM in the basic diet of *L. vannamei*. The results showed that the FCR was decreased and the weight gain rate, specific growth rate, and survival rate of *L. vannamei* were improved. The antioxidant capacity and the activities of immune factors, such as the activities of lysozyme, were improved and the resistance to *V. parahaemolyticus* infection was enhanced. The content of calcium ($Ca^{2+}$) and phosphorus ($PO_4^{3-}$) in blood and muscle of *L. vannamei* was also significantly promoted. Furthermore, MFSM can regulate the structure of intestinal microbiota and metabolites related to antimicrobial substances, inflammatory regulation, and antioxidant functions.

## Author contributions

**Pan Wang:** Investigation, Data curation, Writing—Original draft; **Shanshan Wang:** Investigation, Data curation; **Chuanzhong Zhu:** Conceptualization, Methodology; **Yunzhang Sun:** Methodology; **Qingpi Yan:** Conceptualization, Supervision, Writing—Review & editing; **Ganfeng Yi:** Conceptualization, Funding acquisition.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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## Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.03.009.

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