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KeAi CHINESE ROOTS GLOBAL IMPACT

Original Research Article

Black soldier fly larvae oil can partially replace fish oil in the diet of the juvenile mud crab (*Scylla paramamosain*)

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ABSTRACT

An 8-week feeding trial was conducted to investigate the effects of replacing dietary fish oil (FO) with black soldier fly larval oil (BSFO) on growth performance, antioxidant and immune response, lipid metabolism and mitochondrial function of the juvenile mud crab. A total of 160 mud crabs (18.58 ± 0.02 g) were randomly distributed into five treatments spread across 160 aquaria. There were 4 replicates per treatment and 8 crabs per replicate. The basal diet (Control) contained 3% fish oil and fish oil was replaced with BSFO at 25%, 50%, 75%, and 100% in the remaining four treatments. The results showed that when the proportion of BSFO replacing FO was less than 50%, there were no significant differences in percent weight gain (PWG), specific growth rate (SGR) and feed efficiency (FE) between the experimental and the control groups ($P > 0.05$); however, PWG and SGR decreased as the percentage of substitution increased from 50% to 100% ($P < 0.01$). When the percentage of substitution was less than 50%, the expression levels of genes related to lipid synthesis and catabolism were significantly up-regulated and down-regulated, respectively ($P < 0.05$). When 25% and 50% FO were replaced with BSFO, the antioxidant and immune responses enhanced ($P < 0.05$), and antioxidant and immune-related enzyme activities and metabolite concentrations in the hemolymph and hepatopancreas significantly increased ($P < 0.05$), and the concentrations of malondialdehyde (MDA) and protein carbonyl (PC), and the apoptosis index in the hepatopancreas significantly decreased ($P < 0.05$). Moreover, mitochondrial function indexes in the hepatopancreas, such as mitochondrial DNA copy number and expression levels of energy metabolism-related genes were significantly up-regulated ($P < 0.05$). Hepatopancreas mitochondria were more abundant in crabs fed diets with 25% and 50% replacement of FO with BSFO, while adenosine triphosphate content was the highest in 25% FO replacement group ($P = 0.003$). In summary, the results of the present study demonstrated that the replacement of FO with BSFO at less than 50% (i.e. in-feed BSFO level of 1.5%) did not negatively affect the growth performance of mud crabs, and could improve the antioxidant capacity, immune response, and enhance mitochondrial function.
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1. Introduction

Aquaculture is one of the fastest-growing food industries, and in order to meet the demand for seafood around the world, aquaculture production has steadily increased due to the downward trends in capture fisheries (Jensen et al., 2014). However, rapid expansion of the industry over the past few decades has resulted in a sharp rise in pollution of the environment (Bohnes et al., 2018). The rapid development of aquaculture depends on the supply of

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feed, and the dependence on fish meal and fish oil (FO) for aqua feed leading to overfishing. If the fishing scale is not well controlled, it will inevitably lead to the decline of marine fishery resources. Fish oil, as a marine fishery resource, can be used as a major lipid source in aquatic animal feed production to support animal growth and health due to its rich nutrient content, such as docosahexaenoic acid (DHA; C22:6n-3) and eicosapentaenoic acid (EPA; C20:5n-3). Due to the limited biosynthetic capacity of marine fish or shrimp and crabs, these fatty acids are vital essential nutrients for mariculture animals (Tocher, 2003; Turchini and Francis, 2009). However, as the world's smaller pelagic fisheries remain relatively constant and available marine fisheries become increasingly limited, global FO production is unable to meet the rapidly growing demand for aquafeeds (Fawole et al., 2021; Qin et al., 2022). This has led to a gradual increase in FO prices and supply shortage. Fish oil substitution is another major research focus of aquatic feed in the world after fishmeal substitution (FAO, 2015; Hixson, 2014).

At present, the raw materials used to replace FO are mainly plant derivatives (such as soybean oil and peanut oil) and animal by-products (such as butter and lard) (FAO, 2015). However, previous studies have shown that soybean meal and soybean oil have similar negative environmental impacts as fish meal and FO (Basto-Silva et al., 2019). Some terrestrial plants, such as soybeans, can be used directly for human consumption, but long-term reliance of aquafeed ingredients from cultivation can lead to environmental damage and unsustainability (Wilfart et al., 2016). As a result, there is very little room for plant-based feed development without increasing water pollution and land use or shifting resource needs from the sea to land (Hua et al., 2019). The growing world population is simultaneously increasing the demand for protein, but the available farmland is limited. It is predicted that there will be more than 9 billion people on the planet by 2050, and traditional protein sources might not be enough to feed them, forcing us to look for other options (Godfray et al., 2010).

The Food and Agriculture Organization has recognized insects as a potential source of animal feed. Insects occupy less land, produce far fewer greenhouse gas emissions, and pollute less water than plants or animals (FAO, 2013; Kouřimská and Adámková, 2016). For the past few decades, insects have emerged as a novel source of lipids and proteins in the diets of fish and crustaceans (Cummins et al., 2017; Mohan et al., 2022). Black soldier fly (BSF, *Hermetia illucens*), whose larvae are scavengers, feed on low-value organic wastes, such as kitchen garbage, animal feces, and plant and animal carcasses, converting them into high-value proteins and lipids (Barbi et al., 2020). Black soldier fly only feeds in the larval stage, has high egg production, fast growth rate, short life cycle, simple feeding conditions, and is not a vector of disease (Muller et al., 2017). The average lipid content of BSF larvae is 24.5%, with a maximum lipid content of 45%. They are high in lauric acid (C12:0), palmitic and oleic acids, primarily composed of saturated fatty acids (SFA) (Surendra et al., 2016). The fatty acid profiles in BSF larvae vary depending on their dietary substrate and are considered a promising feed ingredient (Li et al., 2016; Liland et al., 2017). Because medium-chain fatty acids (MCFA) are more polar than other lipids, they rarely rely on celiac particles and lipoproteins for transport, which does not require modification by carnitine palmitoyltransferase-1 (CPT-1) to cross the mitochondrial membrane (the rate-limiting step) (Longo et al., 2016). The addition of MCFA to aquatic feed is used more as an energy source due to the fact that MCFA are absorbed and catabolized more rapidly and are preferentially used for beta oxidation (Fawole et al., 2021), which reduces the consumption of n-3 long-chain poly unsaturated fatty acids (LC-PUFA) and has a “n-3 sparing effect” that promotes the retention of these health-promoting fatty acids (Huang et al., 2021). Lauric acid, with good oxidative stability, is a saturated MCFA containing 12 carbon atoms and has antimicrobial activity, antidiabetic and anti-inflammatory effects, and cardiovascular disease reduction properties (Huang et al., 2023; Luo et al., 2022). Recently, there has been a gradual rise in research on the application of black soldier fly larvae oil (BSFO) to aquafeeds. It was reported that feeding BSFO diets did not affect growth and reduced lipid deposition in the abdominal cavity of Jian carp (*Cyprinus carpio* var. Jian) (Li et al., 2016). Black soldier fly larvae oil is a better lipid source than yellow mealworm or silkworm oil in the feed of young mirror carp (*C. carpio* var. specularis), which can improve their antioxidant capacity and promote growth (Xu et al., 2020b). Fish oil can be replaced by BSFO in feed without negatively affecting the growth, proximate compositions in tissues, or expression levels of genes associated with the fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) (Fawole et al., 2021).

The mud crab (*Scylla paramamosain*) is an economically important marine crustacean widely farmed in the Indian ocean and along the coast of southeastern China (Gong et al., 2022). In China, the farmed mud crab production in 2022 was 154,661 tonnes, which accounted for more than half of the total output of cultured sea crabs in the country (China Fishery Statistical Yearbook, 2023). Because of its delicious flesh, great nutritional content, rapid growth, and significant economic value, it is very well-liked by farmers and consumers (Jiang et al., 2014). It is important to conduct research on low-cost, sustainable, and nutrient-balanced mud crab diets (Zhao et al., 2015). Thus, the aim of the present study was to investigate the effects of replacing FO with BSFO on the growth, fatty acid deposition, antioxidant capacity, immune response, lipid metabolism and mitochondrial function of mud crabs.

2. Materials and methods

2.1. Animal ethics statement

The Ningbo University committee on the ethics of animal experiments (No. SYXK20190005) was founded in 2019. Its jurisdiction was limited to mice, rats, and rabbits, but aquatic species were not included. Crab specimens for the current study came from commercial aquaculture facilities, and all related experimental methods were conducted in strict accordance with the legal guidelines provided by the Zhejiang Provincial Government's Regulation on the Use of Experimental Animals (Order No. 263, issued on August 17, 2009, and in effect as of October 1, 2010). Moreover, these protocols were carried out in conformity with China's Experimental Animal Management Law, guaranteeing adherence to industry benchmarks for animal care and experimentation.

2.2. Diet preparation

Five isonitrogenous (46% protein) and isolipidic (10% lipid) experimental diets were made to satisfy the protein and lipid requirements of juvenile mud crabs. Fish oil was replaced by BSFO at 0, 25%, 50%, 75%, and 100%, respectively. The formulation and ingredients are presented in Table 1, and the fatty acid profiles of FO, BSFO and the experimental diets are shown in Table 2. Black soldier fly larvae oil was obtained by pressing the dried black soldier fly larvae (oil content 41.5%) raised on kitchen waste (provided by Bioforte Biotechnology Co., Ltd., Shenzhen, China). First, the individual ingredients were ground into a fine powder, sifted through an 80-mesh screen, and then weighed according to the formulation. The micro components, such as mineral and vitamin premix, were appropriately mixed by the stepwise enlargement method, and then lipids and purified water were added. The mixture was

Table 1
Ingredients and proximate composition of the experimental diets (%, air-dry basis, unless otherwise stated).

Item	Replacement of FO with BSFO				
	0	25%	50%	75%	100%
Ingredients					
Peru fish meal[1]	30.00	30.00	30.00	30.00	30.00
Krill meal[2]	3.00	3.00	3.00	3.00	3.00
Soybean meal[3]	20.00	20.00	20.00	20.00	20.00
Soybean protein concentrate[4]	12.00	12.00	12.00	12.00	12.00
Wheat flour[5]	23.50	23.50	23.50	23.50	23.50
Vitamin premix[6, 11]	0.50	0.50	0.50	0.50	0.50
Mineral premix[7, 11]	1.00	1.00	1.00	1.00	1.00
$Ca(H_2PO_4)_2$[8]	2.00	2.00	2.00	2.00	2.00
Butylated hydroxytoluene[8]	0.20	0.20	0.20	0.20	0.20
Cholesterol[8]	0.50	0.50	0.50	0.50	0.50
Choline chloride[8]	0.30	0.30	0.30	0.30	0.30
Sodium alginate[8]	2.00	2.00	2.00	2.00	2.00
Soybean lecithin[9]	2.00	2.00	2.00	2.00	2.00
Fish oil[9]	3.00	2.25	1.50	0.75	0.00
BSFO[10]	0.00	0.75	1.50	2.25	3.00
Total	100.00	100.00	100.00	100.00	100.00
Analyzed proximate composition					
Dry matter,% wet weight	93.26	93.27	93.39	93.70	93.76
Crude protein	46.45	46.56	46.76	47.00	46.77
Crude lipid	10.50	10.52	10.36	10.44	10.64
Crude ash	10.54	10.78	10.32	10.78	10.76

FO = fish oil; BSFO = black soldier fly larval oil.
[1] Peru fish meal made in Peru: crude protein, 70.73%, crude lipid, 11.57%.
[2] Krill meal made in Norway: crude protein, 54.91%, crude lipid, 19.89%.
[3] Soybean meal purchased from Tech-Bank Feed Co., Ltd., Ningbo, China: crude protein, 52.44%, crude lipid 1.87%.
[4] Soybean protein concentrate purchased from Tech-Bank Feed Co., Ltd., Ningbo, China: crude protein, 68.01%, crude lipid, 0.60%.
[5] Wheat flour purchased from Tech-Bank Feed Co., Ltd., Ningbo, China: crude protein, 16.21%, crude lipid 1.11%.
[6] Vitamin premix purchased from Tech-Bank Feed Co., Ltd. (Ningbo, China). The formulation was following (g/kg premix): retinyl acetate, 2,500,000 IU; cholecalciferol, 500,000 IU; all-rac-a-tocopherol, 25,000 IU; menadione, 5.63; thiamine, 11.25; riboflavin, 9.5; ascorbic acid, 95; pyridoxine hydrochloride, 10; cyanocobalamin, 0.02; folic acid, 2; biotin, 0.375; nicotinic acid, 37.5; D-Ca pantothenate, 21.5; inositol, 80; antioxidant, 0.5; corn starch, 696.775.
[7] Mineral premix purchased from Tech-Bank Feed Co., Ltd., Ningbo, China. It provided the following (g/kg premix): $FeC_6H_5O_7$, 4.57; $ZnSO_4\cdot7H_2O$, 9.43; $MnSO_4\cdot H_2O$ (99%), 4.14; $CuSO_4\cdot5H_2O$ (99%), 6.61; $MgSO_4\cdot7H_2O$ (99%), 238.97; KH_2PO_4, 233.2; NaH_2PO_4, 137.03; $C_6H_{10}CaO_6\cdot5H_2O$ (98%), 34.09; $CoCl_2\cdot6H_2O$ (99%), 1.36.
[8] $Ca(H_2PO_4)_2$, butylated hydroxytoluene, cholesterol, choline chloride, and sodium alginate were purchased from Macklin Biochemical Co., Ltd., Shanghai, China.
[9] Soybean lecithin and fish oil purchased from Tech-Bank Feed Co., Ltd., Ningbo, China.
[10] Black soldier fly larvae oil purchased from Bioforte Biotechnology Co., Ltd., Shenzhen, China.
[11] Mineral and vitamin premixes were prepared according to Yuan et al. (2020).

blended thoroughly with a Hobart mixer. The mixture was then extruded into cylindrical shapes with diameters of 2.0 and 5.0 mm using a cold extrusion process method (F-26, South China University of Technology Machine Factory, Guangzhou, China), and then cut into pelletized feed (G-250, South China University of Technology Machine Factory, Guangzhou, China) with lengths of 5.0 and 10.0 mm, respectively, to accommodate the different growth stages of the crabs. The feed was then dried to about 10% moisture at 65 °C and stored at −20 °C for subsequent feeding (Yuan et al., 2019).

2.3. Feeding trial

A total of 160 vigorous juvenile mud crabs (18.58 ± 0.02 g) were obtained from the crab field (Sanmen county, Taizhou city, Zhejiang Province, China) and reared in single crab cells (0.45 m long × 0.30 m wide × 0.20 m high) at the Ningbo University experimental base (Ningbo city, Zhejiang Province, China).

Table 2
Fatty acid composition of lipid sources and experimental diets (mg/g, based on dry matter).

Fatty acids	Lipid sources		Replacement of FO with BSFO				
	FO	BSFO	0	25%	50%	75%	100%
C12:0	0.54	65.12	0.06	0.53	1.04	1.49	1.98
C14:0	37.45	23.88	3.31	3.27	3.15	3.02	2.95
C16:0	172.67	114.19	14.15	13.86	13.28	12.79	12.55
C18:0	49.26	20.28	3.67	3.50	3.25	3.04	2.91
C20:0	4.29	0.54	0.27	0.23	0.22	0.20	0.16
Σ SFA	263.67	224.01	21.47	21.39	20.94	20.54	20.55
C16:1n	43.44	12.39	3.73	3.54	3.28	3.13	2.87
C18:1n-9	256.29	188.27	14.41	14.12	13.37	12.78	12.18
C20:1n-9	11.03	1.01	0.76	0.67	0.61	0.57	0.43
C22:1n-11	1.88	0.00	0.17	0.16	0.16	0.12	0.11
Σ MUFA	312.64	201.67	19.08	18.49	17.42	16.60	15.59
C18:2n-6	81.45	48.10	3.07	2.81	2.59	2.40	2.11
C18:3n-6	0.86	0.24	0.09	0.08	0.07	0.07	0.06
C20:2n-6	1.93	0.58	0.14	0.13	0.11	0.10	0.08
C20:4n-6 (ARA)	10.70	2.67	0.55	0.51	0.45	0.41	0.35
Σ n-6 PUFA	94.94	51.59	3.85	3.53	3.22	2.98	2.60
C18:3n-3	25.72	14.64	0.89	0.81	0.78	0.65	0.58
C18:4n-3	7.69	0.50	0.67	0.63	0.55	0.54	0.47
C20:4n-3	3.78	0.50	0.27	0.24	0.23	0.19	0.16
C20:5n-3 (EPA)	44.66	3.10	6.83	6.62	6.26	5.91	5.80
C22:5n-3	9.00	0.17	0.92	0.88	0.79	0.71	0.68
C22:6n-3 (DHA)	122.43	0.25	8.21	7.59	6.57	5.49	4.66
Σ n-3 PUFA	213.28	19.16	17.78	16.77	15.18	13.49	12.35
Total fatty acids	884.53	496.43	62.17	60.18	56.76	53.62	51.09

FO = fish oil; BSFO = black soldier fly larval oil; SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acids; ARA = arachidonic acid; PUFA = polyunsaturated fatty acids; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

Seawater was sourced directly from the ocean and was continuously purified by a series of seawater treatment equipment, including microfiltration, protein separators, biofilters, temperature-regulating tanks, ultraviolet lamps, and oxygenation units. The mud crabs were randomly stocked and sorted into 160 individual crab cells. There were 5 treatments with 4 replicates in each feed, and 8 crabs in each replicate. The ratio of male to female was 1:1, and each replicate was randomly assigned to each feed. Before the start of the feeding experiment, the crabs were acclimated to the experimental environment for one week and fed commercial diets (approximately 45% protein and 8% lipid; Ningbo Tech Bank Feed Co., Ltd., Ningbo, China). Crabs were fed 8% of body weight at the start of the trial, reduced to 7% of body weight after 2 weeks, and 6% of body weight after 4 weeks. These feeding rates ensured that the crabs were well fed. Crabs were fed at 18:00 every day. Uneaten feed, feces, and molted crab shells were removed from the crab cells at 08:00 every day to keep the environment clean and prevent microalgae growth. In addition, any dead crabs were removed, weighed, and recorded daily. The following parameters were applied to the environment in the cells: 20% of the seawater was exchanged at 10:00, the quality of the seawater remained basically constant, ammonia nitrogen less than 0.05 mg/L, temperature between 23 and 28 °C, salinity between 23 and 26 g/L, and dissolved oxygen between 6.3 and 6.9 mg/L. The photoperiod was 10 L:14 D (photo phase from 07:00-17:00), and the light intensity was 200 lx.

2.4. Sample collection

At the end of the feeding experiment, the crabs were counted and weighed in batches to determine their growth performance. Anesthesia was administered in an ice bath, and hemolymph was collected from the heart of each crab using a 2-mL syringe and transferred into a 2-mL centrifuge tube. After resting overnight at

4 °C, the hemolymph was centrifuged at 1200 × g at 4 °C for 10 min in a high-speed centrifuge (Eppendorf Centrifuge 5810R, Germany). After collection, the hemolymph was separated and stored at −80 °C for analysis of biochemical and antioxidant immune parameters. Hepatopancreas samples of crabs after hemolymph collection were rapidly collected into 4-mL centrifuge tubes, and about 2 g and 300 mg hepatopancreas samples of each replicate group were collected into 2- and 1.5-mL (including RNA storage) centrifuge tubes, respectively, and immediately placed in liquid nitrogen and stored at −80 °C for determination of approximate composition, fatty acid composition, biochemical parameters, and gene expression. A small part of the hepatopancreas was placed in a 2-mL centrifuge tube containing 4% fixative solution for histological analysis. Another small portion was placed in a 2-mL (containing glutaraldehyde 2.5%) centrifuge tube at 4 °C for transmission electron microscopy (TEM) analysis. Similarly, muscle samples were collected from each crab into 4-mL centrifuge tubes in liquid nitrogen and stored at −80 °C until the determination of proximate composition.

2.5. Analysis methods

2.5.1. Proximate composition

The analysis methods of the proximate composition of diets, hepatopancreas and muscle were referred to the Association of Official Analytical Chemists (AOAC, 2006). In brief, dry matter was determined by oven drying them at 105 °C until a consistent weight was achieved (method 922.08). Protein was analyzed using the Dumas combustion method using a protein determinator (FP-528, Leco, USA) to obtain nitrogen content, which was multiplied by 6.25 to obtain crude protein content (method 968.06). The determination of lipid content was carried out by petroleum ether extraction using a Soxhlet extractor (Soxtec System HT6, Tecator, Sweden) (method 2000.03). Moisture content was determined by drying to a constant mass at 105 °C (method 934.01), and crude ash content was determined by incineration in a muffle oven at 550 °C for 8 h (method 942.05). The differences in the weight of samples before and after experimental processing were used to calculate the dry matter, crude lipid, and ash contents (Yuan et al., 2020).

2.5.2. Fatty acid composition

The absolute quantifications of fatty acids (mg/g) in diets and tissues were determined using the previous methods (Wang et al., 2021). Briefly, 1 mL of internal standard solution (methyl tricosanoate, C23:0, 1 mg/mL) was added into a 12-mL screwed glass tube. Next, 100 mg of the lyophilized sample was added to the tube. Afterwards, the tube was incubated (at 80 °C for 4 h) in a water bath kettle to produce fatty acid methyl ester (FAME) after being vigorously shaken for 1 min by a vortex oscillator (Genius, IKA, Germany). One millilier n-hexane was added once it had cooled to room temperature, and it was then shaken with a vortex oscillator for 1 min. After that, two-dimensional separation was accelerated with 1 mL of ultrapure water, and bubbles were removed by centrifuging the mixture for 1 min. The supernatant was filtered through a 0.22-μm organic phase ultrafiltration membrane and transferred to a threaded screw-neck vial. A Termovap sample concentrator was used to dry the fatty acid methyl ester (FAME) solution in the vial. The FAMEs were then resuspended using 500 μL of n-hexane, and the results were examined using a gas chromatography-mass spectrometry (GC−MS, 7890B-5977A, Agilent Technologies, USA) equipped with an ultra inert capillary column (DB-WAX fused silica, 30 m × 250 μm internal diameter, film thickness 0.25 μm, Agilent J&W Scientific, USA). The ratios of FAME peak areas to C23:0 peak areas were used to calculate the specific fatty acid concentrations in tissue and diet.

2.5.3. Hemolymph analysis

Hemolymph sample was subjected to measurements of total cholesterol (TCHO), triglycerides (TG), and total protein (TP) using an automated biochemistry analyzer (VITALAB SELECTRA Junior Pros, Netherlands). Enzyme linked immunosorbent assay (ELISA) assay kit from Venning Bio. Inc., Ningbo, China, was used to analyze phenoloxidase (PO; Cat. NO. KT952268-A). Commercial assay kits for the detection of aspartate aminotransferase (AST; Cat. No. C010-1-1), alanine aminotransferase (ALT; Cat. No. C009-2-1), and catalase (CAT; Cat. No. A007-1-1) were obtained from Jiancheng Bio. Inst., Nanjing, China. Operating procedures strictly followed the manufacturer's specifications. The light absorption values were measured by a multimode microplate reader (SpectraMax M2, Thermo). The activities of ALT and AST in the hemolymph were detected by Reitman-Frankel and microplate tests at an absorption peak of 510 nm. Catalase activity was analyzed using the kit by the ammonium molybdate method at an absorption peak of 405 nm.

2.5.4. Oxidation and antioxidants, immune parameters detection

Hemolymph samples were centrifuged at 4 °C and 1200 × g for 10 min, the supernatant was isolated, and the biochemical indexes were analyzed. Hepatopancreas samples were homogenized by a homogenizer (IKA T25 digital Ultra-Turrax, Germany) with cold 0.86% saline (1:9, v/w) and then by a microcentrifuge (Eppendorf 5810R; 1200 × g at 4 °C for 10 min). The supernatant was transferred into a 200-μL microcentrifuge tube for biochemical determination. Malondialdehyde (MDA; Cat. No. A003-1-2), protein carbonyl (PC; Cat. No. A087-1-2), glutathione (GSH; Cat. No. A006-2-1), total antioxidant capacity (T-AOC; Cat. No. A015-2-1), glutathione peroxidase (GSH-Px; Cat. No. A005-1-2), total protein (TP; Cat. No. A045-2-2), superoxide dismutase (T-SOD; Cat. No. A001-1-2), nitric oxide (NO; Cat. No. A012-1-2), nitric oxide synthase (NOS; Cat. No. A014-2-2), and lysozyme (LZM; Cat. No. A050-1-1) in the hepatopancreas and hemolymph were analyzed using assay kits (Jiancheng Bio. Inst.). Operating procedures strictly followed the manufacturer's specifications. The light absorption values were measured by a multimode microplate reader (SpectraMax M2, Thermo).

Total protein content was detected by the Cauldron Brilliant Blue method at an absorption peak of 595 nm. The concentration of MDA was analyzed by the thiobarbituric acid (TBA) method. The red product was formed by the condensation of MDA and TBA in lipid peroxidation degradation products, with a maximum absorption peak at 532 nm. Protein carbonyl reacts with 2,4-dinitrophenylhydrazine to form a red 2,4-dinitrophenylhydrazone precipitate, and the product has a characteristic absorption peak at 370 nm. Total antioxidant capacity (T-AOC) was analyzed using the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method at an absorption peak of 405 nm. The activity of T-SOD was analyzed by the hydroxylamine method using the diagnostic reagent kit. Reaction product concentration was proportional to the amount of generated superoxide anion radical, which caused the increasing absorbance at 550 nm. Glutathione peroxidase (GSH-Px) activity was analyzed by the colorimetric method at an absorption peak of 412 nm. The concentration of glutathione was analyzed with reference to previous methods, glutathione reacts with dithiodinitrobenzoic acid (DTNB) to form a yellow product with an absorbance of 420 nm. Nitrate reductase determines the concentration of NO and specifically reduces NO_3^- to NO_2^-, the concentration of which was determined by the color depth. Nitric oxide synthase (NOS) catalyzes L-arginine to react with molecular oxygen to produce NO, and NO reacts with nucleophilic substances to produce colored compounds. Absorbance was measured at 530 nm wavelength. The activity of lysozyme was analyzed by turbidimetry at the absorption peak of 530 nm. Because lysozyme has the ability

to lyse bacteria, the concentration was reduced and the transmittance was improved.

2.5.5. Enzyme activities related to lipid metabolism

ELISA assay kits (Venning Bio. Inc., Ningbo, China) were used to analyze the activities of fatty acid synthase (FAS; Cat. No. KT95244-A) and carnitine palmitoyltransferase (CPT; Cat. No. KT20804-A). The steps of operation strictly followed the manufacturer's protocol. The light absorption values were measured by a multi-mode microplate reader (SpectraMax M2, Thermo).

2.5.6. Indexes related to mitochondrial function

The concentration of adenosine triphosphate (ATP) (Cat. No. A095-1-1) in the hepatopancreas and the activity of succinate dehydrogenase (SDH; Cat. No. A022-1-1) were analyzed colorimetrically at 636 and 600 nm, respectively, using analytical kits (Jiancheng Bio. Inst.). As was previously indicated, Yuan et al. (2022) approach was used to determine the mitochondrial DNA copy number (mtDNAcn) in the hepatopancreas. In brief, genomic DNA was extracted from the hepatopancreas samples of mud crabs using the Universal Genomic DNA Purification Mini Spin Kit (Cat. No. D0063; Beyotime, Shanghai, China). The steps of operation followed the manufacturer's protocol strictly. The relative mtDNA copy number was determined by quantitative real time PCR with primers for the mitochondrial 16S rRNA gene and the nuclear β-actin gene. The primers used for quantitative PCR (qPCR) are listed in Table S1, and the qPCR procedure is described below.

2.5.7. Real-time quantitative PCR

Real-time qPCR techniques were used to ascertain the expression levels of genes associated with energy metabolism, mtDNAcn, antioxidant capability, and lipid metabolism. The process and cycle conditions were previously described by Luo et al. (2020). In short, TRIzol and HiScript RT SuperMix kits (Vazyme, Nanjing, China) were used to extract and reverse transcribe RNA, respectively. The target genes' relative expression levels were computed using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The house-keeping gene used was β-actin, and the control/reference group was the 0 BSFO replacement group.

2.5.8. Transmission electron microscope

Hepatopancreatic transmission electron microscopy (TEM) analysis was performed utilizing the prior technique with slight modifications (Shi et al., 2021). Fresh mud crab hepatopancreas sample was immediately fixed in 2.5% glutaraldehyde and then post-fixed for 1 to 2 h with 1% osmium tetroxide (SPI-Chem, USA). Ultrathin sections (80-nm-thick) were cut and dehydrated in ethanol at 30%, 50%, 70%, 90%, then in 90% acetone, and finally in 100% anhydrous acetone (15 min per step). The penetrated sections were immersed in acetone and SPI-Pon812 embedding agent (SPI, USA) for 1 h each time. A pure embedding agent was added, and the polymerization process was carried out at 37 °C for 12 h and 60 °C for 36 h. After that, slices were stained with lead citrate (Merck KGaA, Germany) and uranyl acetate (Ted Pella, USA), and finally the ultrastructure was examined by TEM (Hitachi H-7, 650, Japan).

2.5.9. Hematoxylin and eosin (H&E) section and observation

The crabs were immediately dissected, and a fresh hepatopancreatic sample was fixed in 4% tissue fixative (paraformaldehyde) to make paraffin sections. The hepatopancreatic sample was prepped for dehydration by ethanol. The concentration of which was raised from 75% to 100% after fixation (for at least 24 h). The sample was then divided into 4-μm sections and fixed in paraffin. Finally, after staining with H&E, the height of resorptive cells in the hepatopancreas was observed and measured using a microscope (Olympus, DP72) and ImagePro software.

2.5.10. Immunofluorescence

One step TUNEL apoptosis assay kit (Beyotime, China) was used to analyze apoptosis. In brief, the paraffin sections underwent a series of procedures, including xylene dewaxing, gradient ethanol solution rehydration, proteinase K treatment for 5 min at 25 °C, and 5 min of phosphate buffered solution (PBS) washing. The treated samples were first cleaned with PBS, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (C1006, Beyotime, China). Finally, they were stained for 60 min at 37 °C in the dark using a mixture of terminal deoxynucleotidyl transferase and fluorescein-12-2-deoxy-uridine-5′triphosphate. The slices were examined using a laser scanning confocal microscope (LSM880, Carl Zeiss Microscopy GmbH, Germany) after being sealed with antifade mounting medium (Beyotime, China).

Photographs and analyses at magnification 200× were taken on slices from three crabs per treatment to determine the degree of hepatopancreas apoptosis. TUNEL was used to stain the apoptotic cells green, and DAPI was used to color all the nuclei blue. Each section's five randomly chosen sections were examined using ImageJ software. Using the counting menu, the green and blue fluorescence in the seen area was marked and tallied. The ratio was then used to construct the apoptosis index.

2.6. Calculations and statistical analysis

The calculation formulae were as follows:

$$\text{Percent weight gain (PWG, \%)} = 100 \times \frac{W_t - W_i}{W_i},$$

$$\text{Specific growth rate (SGR, \% / day)} = 100 \times \frac{\ln W_t - \ln W_i}{t},$$

$$\text{Feed efficiency (FE)} = \frac{W_a + W_b - W_c}{Q},$$

$$\text{Survival (\%)} = 100 \times \frac{N_t}{N_i},$$

where W_t (g) represents the final average weight, W_i (g) represents the initial average weight, t represents the days of feeding, W_a (g) represents the total final crab weight, W_b (g) represents the total dead crab weight, W_c (g) represents the total initial crab weight, Q represents the total feed intake (g), N_t (g) represents the final crab number, N_i (g) represents the initial crab number.

A one-way analysis of variance (ANOVA) was performed on all data using SPSS 22.0 (SPSS Inc., Chicago, IL, USA), followed by Tukey's multiple range test. Using orthogonal polynomial contrasts, a follow-up trend analysis was performed to determine if the significant effects were quadratic or linear. A difference was deemed significant if the P-value was less than 0.05. A difference was considered highly significant if the P-value was less than 0.01.

The statistical model used for the ANOVA was as follows:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

where Y_{ij} represents the observed value for the dependent variable, μ is the overall mean, representing the grand mean of all observations, α_i is the effect of the i-th treatment (i.e., different dietary BSFO levels), and ε_{ij} is the random error term associated with the j-th observation under the i-th treatment.

3. Results

3.1. Growth performance and feed utilization

As shown in Table 3, dietary replacement of FO with BSFO was significantly negatively correlated with final weight (FW) ($P = 0.009$), percent weight gain (PWG) ($P = 0.006$), and specific growth rate (SGR) ($P = 0.006$). When the proportion of BSFO replacing FO in the diet was less than 50%, FW, PWG and SGR were not significantly different($P > 0.05$); however, when the replacement level of FO with BSFO exceeded 50%, the FW, PWG and SGR decreased significantly ($P < 0.01$). Crabs fed diets with 75% and 100% replacement of FO with BSFO showed lower FE than those fed the other diets ($P = 0.010$). The lowest survival was observed in crabs fed the diet with 100% replacement of FO with BSFO ($P = 0.012$).

3.2. Proximate composition in tissues

There were no significant differences in the contents of moisture ($P = 0.135$), protein ($P = 0.123$), lipid ($P = 0.418$), and ash ($P = 0.170$) in muscle among all treatments (Table 4). Crabs fed diets with the control and 25% replacement of FO with BSFO exhibited higher protein content in the hepatopancreas than those fed the other diets ($P = 0.001$); however, crabs fed diets with control and 25% replacement of FO with BSFO showed lower lipid content in the hepatopancreas than those fed the other diets ($P = 0.001$). The contents of moisture ($P = 0.130$) and ash ($P = 0.152$) in hepatopancreas were not significantly influenced by dietary replacement of FO with BSFO.

3.3. Fatty acid profiles in tissues

The fatty acid profile and the principal component analysis (PCA) score plot and heat map visualization of the hepatopancreas are shown in Table 5 and Fig. 1, respectively. Lauric acid (C12:0) in the hepatopancreas significantly increased with dietary replacement of FO with BSFO increasing from 0 to 100% (Table 5, $P < 0.001$). The variance of the two main components of the hepatopancreas fatty acids, PC1 and PC2, was 73.6% (Fig. 1; 59.0% and 14.6% of the total variance, respectively). Saturated fatty acids (SFA) ($P = 0.001$), mono-unsaturated fatty acids (MUFA) ($P < 0.001$), and total fatty acids ($P = 0.006$) in the hepatopancreas significantly increased with an increase of dietary replacement of FO with BSFO. Among all treatments, there were no significant differences in n-3 polyunsaturated fatty acids (n-3 PUFA) ($P = 0.440$), C20:5n-3 (EPA) ($P = 0.093$), and C22:6n-3 (DHA) ($P = 0.076$). In contrast, as the replacement level of FO with BSFO increased, the ratio of DHA to EPA significantly decreased ($P = 0.012$).

The macroscopic effects of dietary replacement of FO with BSFO on fatty acid composition in hepatopancreatic were visualized by heat mapping (Fig. 1). Red indicated higher values and blue indicated lower values. Fatty acid contents in the hepatopancreas significantly increased with the increase of replacing of FO with BSFO ($P < 0.05$). As the substitution level of FO with BSFO exceeded 50%, there were more red squares in the heat map, which indicated a rise in fatty acid content.

The fatty acid profile and the principal component analysis (PCA) score plot and heat map visualization of the muscle are presented in Table 6 and Fig. 2. The concentration of lauric acid in muscle was not affected by dietary replacement of FO with BSFO ($P = 0.452$). The two main components of fatty acids in muscle (PC1 and PC2) had a variance of 64.5% (Fig. 2; 46.8% and 17.7% of the total variance, respectively). The contents of SFA ($P = 0.015$), MUFA ($P < 0.001$), n-3 PUFA ($P = 0.004$), n-6 PUFA ($P < 0.001$), and total fatty acids ($P = 0.002$) in muscle showed linear and quadratic effects, and the highest value was found in crabs fed diet with 25% replacement of FO with BSFO. The macroscopic effects of dietary replacement of FO with BSFO on fatty acid composition in muscle were visualized by heat mapping (Fig. 2).

Table 3
Growth performance and feed utilization of mud crab fed with different experimental diets for 8 weeks.

Item	Replacement of FO with BSFO					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
IW, g	18.57	18.55	18.54	18.62	18.64	0.023	0.619	0.102	0.310
FW, g	38.17[b]	37.89[b]	37.12[b]	34.99[a]	34.13[a]	0.439	0.009	0.000	0.001
PWG, %	105.63[b]	104.26[b]	100.24[b]	87.97[a]	83.18[a]	2.426	0.006	0.000	0.001
Survival, %	78.13[b]	78.13[b]	75.00[b]	71.88[b]	59.38[a]	2.198	0.012	0.001	0.001
SGR, %/d	1.29[b]	1.27[b]	1.18[b]	1.12[a]	1.08[a]	0.022	0.006	0.000	0.001
FE	0.82[b]	0.81[b]	0.75[ab]	0.67[a]	0.64[a]	0.022	0.010	0.000	0.001

FO = fish oil; BSFO = black soldier fly larval oil; IW = initial weight; FW = final weight; PWG = percent weight gain; SGR = specific growth rate; FE = feed efficiency.
Data are expressed as the means ($n = 4$).
[a,b] Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 4
Proximate composition of the muscle and hepatopancreas of juvenile mud crab fed with different experimental diets for 8 weeks (%, wet weight).

Item	Replacement of FO with BSFO					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
Hepatopancreas									
Moisture	65.52	67.75	66.65	65.70	66.79	0.312	0.130	0.832	0.732
Protein	15.80[c]	13.98[b]	12.46[a]	12.78[ab]	12.62[a]	0.353	0.000	0.000	0.000
Lipid	12.00[ab]	11.02[a]	12.17[bc]	12.69[c]	12.35[ab]	0.171	0.001	0.007	0.030
Ash	1.46	1.35	1.42	1.47	1.39	0.016	0.152	0.822	0.959
Muscle									
Moisture	78.45	78.00	76.60	75.91	76.04	0.402	0.135	0.009	0.029
Protein	18.38	19.27	20.18	20.78	20.94	0.350	0.123	0.005	0.019
Lipid	0.85	0.75	0.88	0.92	0.90	0.029	0.418	0.214	0.446
Ash	1.57	1.61	1.76	1.73	1.76	0.030	0.170	0.022	0.056

FO = fish oil; BSFO = black soldier fly larval oil.
Data are expressed as the means ($n = 3$).
[a–c] Values in the same row with different superscripts are different ($P < 0.05$).

Table 5
Fatty acid composition of the hepatopancreas of mud crab fed with different experimental diets for 8 weeks (mg/g, dry hepatopancreas).

Fatty acids	Replacement of FO with BSFO					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
C12:0	0.07^{a}	1.05^{b}	2.04^{c}	2.57^{c}	4.64^{d}	0.416	0.000	0.000	0.000
C14:0	5.64^{a}	6.41^{ab}	6.38^{ab}	6.71^{bc}	7.34^{c}	0.163	0.002	0.000	0.000
C16:0	30.34^{a}	31.95^{ab}	32.75^{ab}	32.60^{ab}	34.40^{b}	0.460	0.049	0.002	0.012
C18:0	9.49^{a}	9.92^{ab}	10.66^{ab}	10.98^{ab}	11.77^{b}	0.278	0.045	0.001	0.005
C20:0	0.69	0.69	0.73	0.71	0.72	0.013	0.811	0.375	0.603
Σ SFA	46.22^{a}	50.03^{ab}	52.56^{abc}	53.56^{bc}	58.86^{c}	1.231	0.001	0.000	0.000
C16:1n	8.33	9.25	9.35	9.98	10.03	0.235	0.119	0.007	0.024
C18:1n-9	40.14^{a}	43.23^{ab}	45.46^{b}	45.68^{b}	51.76^{c}	1.071	0.000	0.000	0.000
C20:1n-9	2.40	2.35	2.41	2.47	2.38	0.046	0.963	0.792	0.948
C22:1n-11	0.74	0.75	0.85	0.63	0.85	0.043	0.545	0.758	0.928
Σ MUFA	51.61^{a}	55.59^{ab}	58.07^{b}	58.76^{b}	65.02^{c}	1.253	0.000	0.000	0.000
C18:2n-6	18.74^{a}	19.57^{ab}	19.05^{ab}	18.09^{a}	21.28^{b}	0.382	0.018	0.157	0.119
C18:3n-6	0.15^{a}	0.17^{ab}	0.19^{ab}	0.20^{ab}	0.20^{b}	0.006	0.034	0.001	0.015
C20:2n-6	1.42^{a}	1.41^{a}	1.84^{b}	1.50^{ab}	1.82^{b}	0.059	0.006	0.028	0.096
C20:4n-6	2.59^{a}	2.53^{a}	3.75^{b}	3.97^{b}	3.63^{ab}	0.187	0.004	0.003	0.006
Σ n-6 PUFA	22.91^{a}	23.69^{ab}	24.82^{ab}	23.76^{ab}	26.93^{b}	0.475	0.035	0.009	0.029
C18:3n-3	4.54^{a}	4.97^{ab}	4.98^{ab}	5.16^{ab}	5.62^{b}	0.116	0.022	0.000	0.003
C18:4n-3	1.04	1.11	1.16	1.06	1.19	0.029	0.489	0.247	0.521
C20:4n-3	0.79	0.83	1.15	0.84	0.81	0.050	0.095	0.875	0.188
C20:5n-3	15.33	16.79	17.57	16.41	17.90	0.337	0.093	0.041	0.103
C22:5n-3	2.96	2.98	3.37	3.31	3.41	0.072	0.089	0.010	0.035
C22:6n-3	20.96	21.02	20.42	18.85	18.90	0.351	0.076	0.006	0.023
Σ n-3 PUFA	45.62	47.70	48.65	45.62	47.86	0.616	0.440	0.601	0.700
Total fatty acids	166.36^{a}	177.01^{a}	184.10^{ab}	181.70^{ab}	198.81^{b}	3.276	0.006	0.000	0.002
EPA + DHA	36.29	37.81	37.99	35.26	36.91	0.463	0.341	0.656	0.711
DHA/EPA	1.37^{b}	1.26^{ab}	1.16^{ab}	1.15^{ab}	1.05^{a}	0.034	0.012	0.000	0.001

FO = fish oil; BSFO = black soldier fly larval oil; SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.
Data are expressed as the means ($n = 3$).
[a-d] Values in the same row with different superscripts are different ($P < 0.05$).

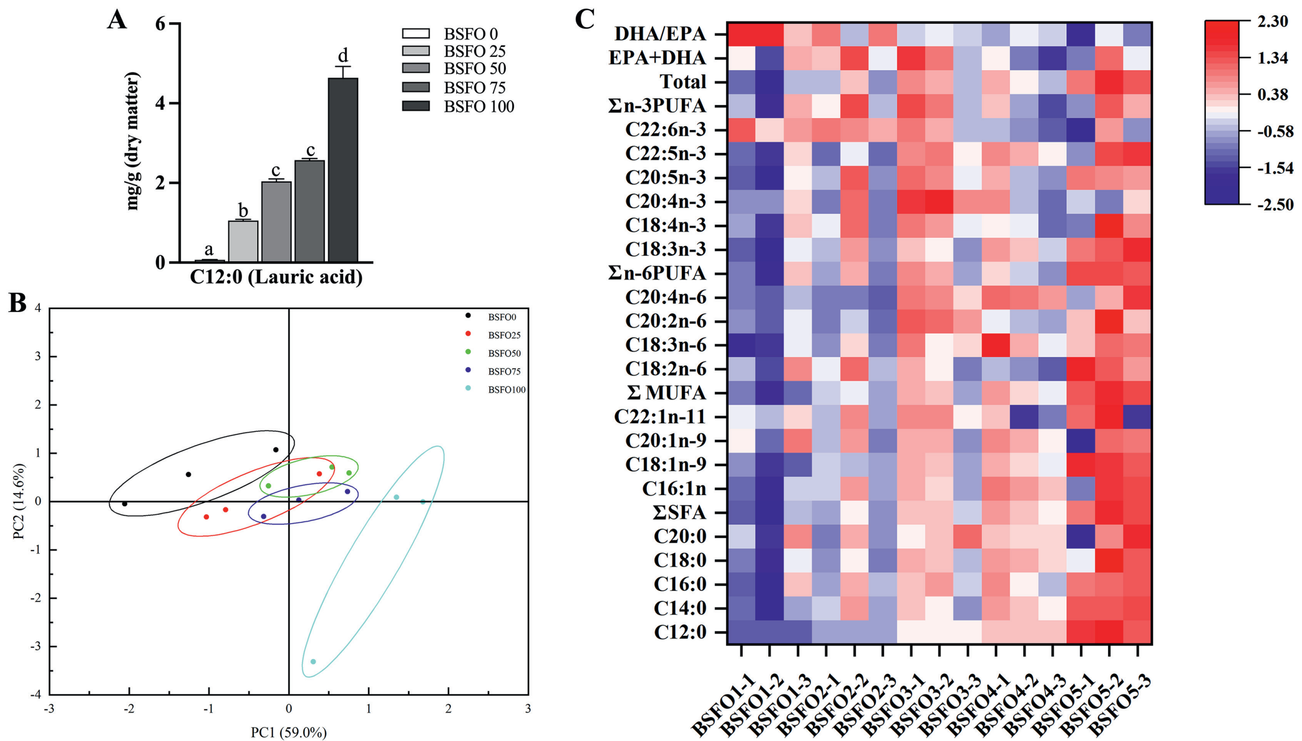

Fig. 1. Effects of dietary replacement of FO with BSFO on fatty acid composition in the hepatopancreas of mud crabs (mg/g dry hepatopancreas). (A) Lauric acid. (B) Principal component analysis (PCA) model of fatty acids. (C) Heat map visualization of fatty acids. Data are expressed as the means ($n = 3$). [a-d] Mean values with unlike letters are significantly different ($P < 0.05$). SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acids; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid. FO = fish oil; BSFO = black soldier fly larval oil.

Table 6
Fatty acid composition in the muscle of mud crab fed with different experimental diets for 8 weeks (based on dry muscle).

Fatty acids	Replacement of FO with BSFO,%					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
C12:0	0.01	0.01	0.01	0.01	0.01	0.000	0.452	0.690	0.093
C14:0	0.11	0.11	0.10	0.10	0.10	0.002	0.097	0.003	0.015
C16:0	2.72^{ab}	2.88^{b}	2.60^{a}	2.62^{a}	2.75^{ab}	0.031	0.005	0.382	0.472
C18:0	2.06^{a}	2.24^{b}	2.13^{ab}	2.04^{a}	2.12^{ab}	0.024	0.020	0.656	0.609
C20:0	0.07^{ab}	0.06^{a}	0.07^{ab}	0.07^{ab}	0.08^{b}	0.002	0.044	0.021	0.033
Σ SFA	4.97^{ab}	5.30^{b}	4.91^{a}	4.85^{a}	5.05^{ab}	0.051	0.015	0.021	0.033
C16:1n	0.40^{b}	0.40^{b}	0.39^{b}	0.40^{b}	0.32^{a}	0.009	0.001	0.445	0.747
C18:1n-9	2.92^{bc}	2.95^{c}	2.73^{a}	2.78^{ab}	2.80^{abc}	0.026	0.004	0.004	0.001
C20:1n-9	0.12^{a}	0.14^{b}	0.13^{b}	0.11^{ab}	0.09^{a}	0.005	0.007	0.019	0.027
C22:1n-11	0.06^{b}	0.05^{ab}	0.05^{a}	0.04^{a}	0.04^{a}	0.002	0.012	0.009	0.002
Σ MUFA	3.51^{b}	3.55^{b}	3.30^{a}	3.33^{a}	3.26^{a}	0.034	0.000	0.002	0.002
C18:2n-6	0.93	0.99	1.03	0.89	0.98	0.017	0.067	0.975	0.743
C20:2n-6	0.24^{c}	0.25^{c}	0.20^{b}	0.17^{a}	0.19^{ab}	0.009	0.000	0.001	0.004
C20:4n-6	1.41^{c}	1.40^{c}	1.38^{bc}	1.26^{b}	1.08^{a}	0.035	0.000	0.000	0.000
Σ n-6 PUFA	2.58^{b}	2.64^{b}	2.61^{b}	2.32^{a}	2.25^{a}	0.046	0.000	0.000	0.000
C18:3n-3	0.20^{a}	0.24^{b}	0.26^{b}	0.18^{a}	0.18^{a}	0.010	0.000	0.109	0.005
C18:4n-3	0.03	0.03	0.03	0.03	0.02	0.001	0.072	0.063	0.090
C20:4n-3	0.04^{bc}	0.05^{c}	0.04^{bc}	0.02^{ab}	0.02^{a}	0.003	0.004	0.003	0.001
C20:5n-3	4.80	5.26	4.53	4.74	4.65	0.092	0.092	0.226	0.483
C22:5n-3	0.32^{c}	0.24^{b}	0.18^{a}	0.18^{a}	0.18^{a}	0.015	0.000	0.000	0.000
C22:6n-3	3.61^{a}	4.093^{b}	3.44^{a}	3.38^{a}	3.47^{a}	0.075	0.000	0.052	0.154
Σ n-3 PUFA	8.99^{a}	9.92^{b}	8.48^{a}	8.53^{a}	8.51^{a}	0.169	0.004	0.044	0.137
Total fatty acids	20.06^{ab}	21.40^{b}	19.29^{a}	19.03^{a}	19.07^{a}	0.269	0.002	0.016	0.057
EPA + DHA	8.41^{ab}	9.36^{b}	7.97^{a}	7.88^{a}	8.12^{a}	0.157	0.004	0.075	0.217
DHA/EPA	0.76	0.78	0.76	0.71	0.75	0.010	0.370	0.231	0.503

FO = fish oil; BSFO = black soldier fly larval oil; SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.
Data are expressed as the means ($n = 3$).
[a-d] Values in the same row with different superscripts are different ($P < 0.05$).

Red indicated higher values and blue indicated lower values. As the substitution level of FO with BSFO exceeded 50%, there were more blue squares in the heat map, which indicated a decrease in fatty acid content.

3.4. Hemolymph biochemical profile

The effects of dietary substitution of BSFO for FO on biochemical indicators in the hemolymph are presented in Table 7. Crabs fed the

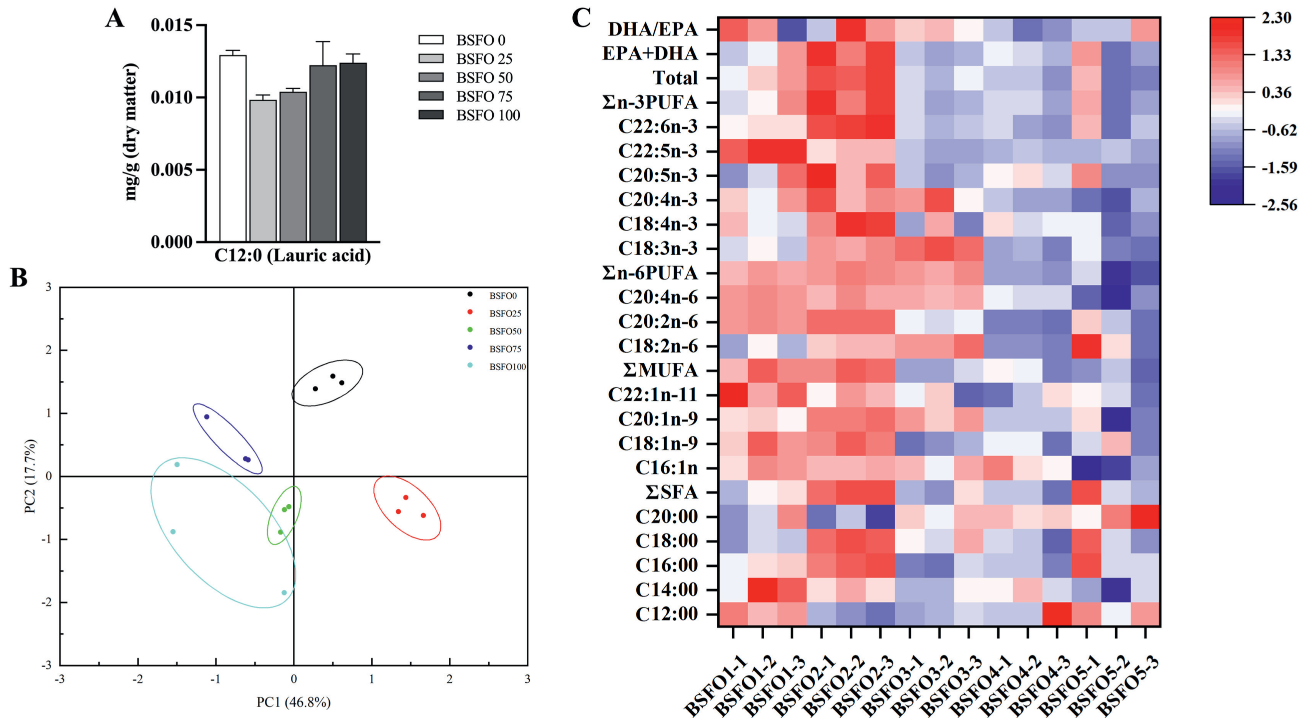

Fig. 2. Effects of dietary replacement of FO with BSFO on fatty acid composition in the muscle of mud crabs (mg/g dry muscle). (A) Lauric acid content. (B) Principal component analysis (PCA) model of fatty acids. (C) Heat map visualization of fatty acids. Data are expressed as the means ($n = 3$). SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acids; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid. FO = fish oil; BSFO = black soldier fly larval oil.

Table 7
The hemolymph biochemical indexes of mud crab fed with different experimental diets for 8 weeks.

Item	Replacement of FO with BSFO					SEM	P-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
TG, μmol/L	0.66[a]	0.86[a]	1.67[b]	0.89[a]	0.79[a]	0.098	0.000	0.684	0.012
TCHO, μmol/L	0.95[a]	0.86[a]	1.58[b]	1.02[a]	1.19[ab]	0.079	0.004	0.249	0.268
ALT, U/L	16.18[a]	17.14[a]	50.80[b]	46.64[b]	49.65[b]	4.455	0.000	0.000	0.000
AST, U/L	26.65	24.06	25.14	31.96	26.17	1.363	0.070	0.338	0.642
CAT, U/mL	8.58[a]	7.23[a]	14.39[b]	12.23[ab]	12.00[ab]	0.831	0.010	0.038	0.066
T-SOD, U/mL	386.23[a]	414.11[ab]	425.43[b]	393.92[ab]	420.45[ab]	4.972	0.016	0.179	0.225
GSH-Px, μmol/L	430.37[a]	455.54[ab]	477.09[b]	441.44[ab]	463.06[ab]	5.608	0.039	0.207	0.173
GSH, μmol/L	4.94[ab]	6.10[b]	2.77[a]	3.98[ab]	2.56[a]	0.432	0.015	0.018	0.066
NO, μmol/L	85.29[a]	132.77[b]	104.44[ab]	111.75[ab]	118.74[ab]	5.082	0.014	0.213	0.267
NOS, U/mL	2.88[a]	2.34[a]	3.66[b]	2.58[a]	2.42[a]	0.140	0.001	0.518	0.331
LZM, μg/mL	3.07[a]	3.07[a]	4.04[b]	2.97[a]	3.42[ab]	0.127	0.004	0.488	0.409
PO, U/L	27.93[a]	33.45[ab]	44.23[b]	37.24[ab]	25.47[a]	2.243	0.026	0.947	0.008

FO = fish oil; BSFO = black soldier fly larval oil; TG = triglyceride; TCHO = total cholesterol; ALT = alanine transaminase; AST = aspartate aminotransferase; CAT = catalase; T-SOD = superoxide dismutase; GSH-Px = glutathione Peroxidase; GSH = glutathione; NO = nitric oxide; NOS = nitric oxide synthase; LZM = lysozyme; PO = phenoloxidase.
Data are expressed as the means ($n = 3$).
[a,b] Values with different letters in the same row were significantly different ($P < 0.05$).

diet with 50% replacement of FO with BSFO showed higher concentrations of TG ($P < 0.001$) and TCHO ($P = 0.004$) in the hemolymph than those fed the control diet. The activities of CAT ($P = 0.010$), GSH-Px ($P = 0.039$), T-SOD ($P = 0.016$), NOS ($P = 0.001$), lysozyme ($P = 0.004$), PO ($P = 0.026$) and ALT ($P < 0.001$) in the hemolymph dramatically increased with an increase of dietary replacement of FO with BFSO, and a maximum was observed in diet with 50% replacement of FO with BSFO. The AST activity in the hemolymph was not significantly different among all treatments ($P = 0.070$). Crabs fed the diet with 25% replacement of FO with BSFO showed higher activity of GSH in the hemolymph than those fed the other diets ($P = 0.015$).

3.5. Hepatopancreatic antioxidant and immune capacity

As shown in Table 8, the crabs fed with the control diet showed the highest concentrations of MDA and PC in the hepatopancreas among all treatments ($P = 0.024$; $P = 0.018$). The highest activities of CAT and GSH in hepatopancreas were observed in crabs fed diet with 50% replacement of FO with BSFO among all treatments ($P = 0.001$; $P = 0.043$). Meanwhile, crabs fed the diet with 25% replacement of FO with BSFO exhibited higher activities of T-SOD and T-AOC in the hepatopancreas than those fed the control diet ($P = 0.018$; $P = 0.008$). However, the concentration of GSH-Px was not significantly affected by dietary replacement of FO with BSFO ($P = 0.238$).

The activity of lysozyme in the hepatopancreas significantly increased with dietary replacement of FO with BSFO ($P < 0.001$). Crabs fed the diet with 50% replacement of FO with BSFO exhibited higher activities of NO ($P = 0.015$), NOS ($P = 0.044$) and PO ($P = 0.023$) in the hepatopancreas than those fed the other diets.

3.6. Apoptosis of hepatopancreas cells

The effect of dietary replacement of FO with BSFO on apoptosis of the hepatopancreas cells is shown in Fig. 3. Of all the groups, the crabs fed diet with 50% replacement of FO with BSFO had the lowest hepatopancreas cell apoptosis index among all treatments (Fig. 3, $P = 0.044$). The expression levels of apoptosis-related genes such as p38 mitogen-activated protein kinases (*p38-mapk*) and bcl-2 associated X protein (*bax*) were markedly down-regulated in the 50% replacement of FO with BSFO ($P = 0.018$; $P = 0.027$). The expression of *bax/bcl-2* ratio in the hepatopancreas was significantly down-regulated when the replacement of FO with BSFO increased from 0 to 50% ($P = 0.022$). However, the expression of genes such as cysteinyl aspartate specific proteinase 2 (*caspase 2*) and B cell lymphoma-2 (*bcl-2*) were not significantly affected by dietary replacement of FO with BSFO (Fig. 3, $P > 0.05$).

Table 8
Antioxidant and immune properties of the hepatopancreas of juvenile mud crab fed with different experimental diets for 8 weeks.

Item	Replacement of FO with BSFO					SEM	P-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
CAT, U/mg prot	90.05[a]	119.77[ab]	145.90[b]	86.96[a]	90.14[a]	6.848	0.001	0.521	0.028
GSH-Px, U/mg prot	484.60	496.94	562.90	489.86	516.34	12.091	0.238	0.530	0.475
GSH, mgGSH/g prot	26.43[a]	34.45[ab]	42.35[b]	28.20[a]	35.10[ab]	1.958	0.043	0.444	0.256
T-SOD, U/mg prot	54.01[a]	71.17[b]	67.91[ab]	63.98[ab]	55.98[ab]	2.181	0.018	0.842	0.007
T-AOC, U/mg prot	5.50[a]	9.74[b]	7.57[ab]	5.80[a]	5.97[a]	0.498	0.008	0.412	0.119
MDA, nmol/mg prot	2.38[b]	1.41[ab]	1.41[ab]	1.65[ab]	0.93[a]	0.164	0.024	0.018	0.067
PC, nmol/mg prot	4.19[b]	4.12[b]	1.76[ab]	2.80[ab]	1.03[a]	0.389	0.018	0.001	0.008
LZM, μg/mg prot	10.76[a]	10.94[ab]	9.10[a]	14.84[bc]	16.73[c]	0.823	0.000	0.240	0.025
NO, μmol/g prot	13.15[a]	15.29[a]	33.67[b]	25.31[ab]	25.84[ab]	2.441	0.015	0.034	0.026
NOS, μg prot/L	58.61[a]	58.00[a]	66.61[ab]	84.42[b]	70.48[ab]	3.344	0.044	0.027	0.076
PO, U/g prot	14.97[ab]	15.50[ab]	17.46[b]	13.15[a]	12.86[a]	0.562	0.023	0.096	0.045

FO = fish oil; BSFO = black soldier fly larval oil; CAT = catalase; GSH-Px = glutathione Peroxidase; GSH = glutathione; T-SOD = superoxide dismutase; T-AOC = total antioxidant capacity; MDA = malondialdehyde; PC = protein carbonyl; LZM = lysozyme; NO = nitric oxide; NOS = nitric oxide synthase; PO = phenoloxidase.
Data are expressed as the means ($n = 3$).
[a–c] Values with different letters in the same row were significantly different ($P < 0.05$).

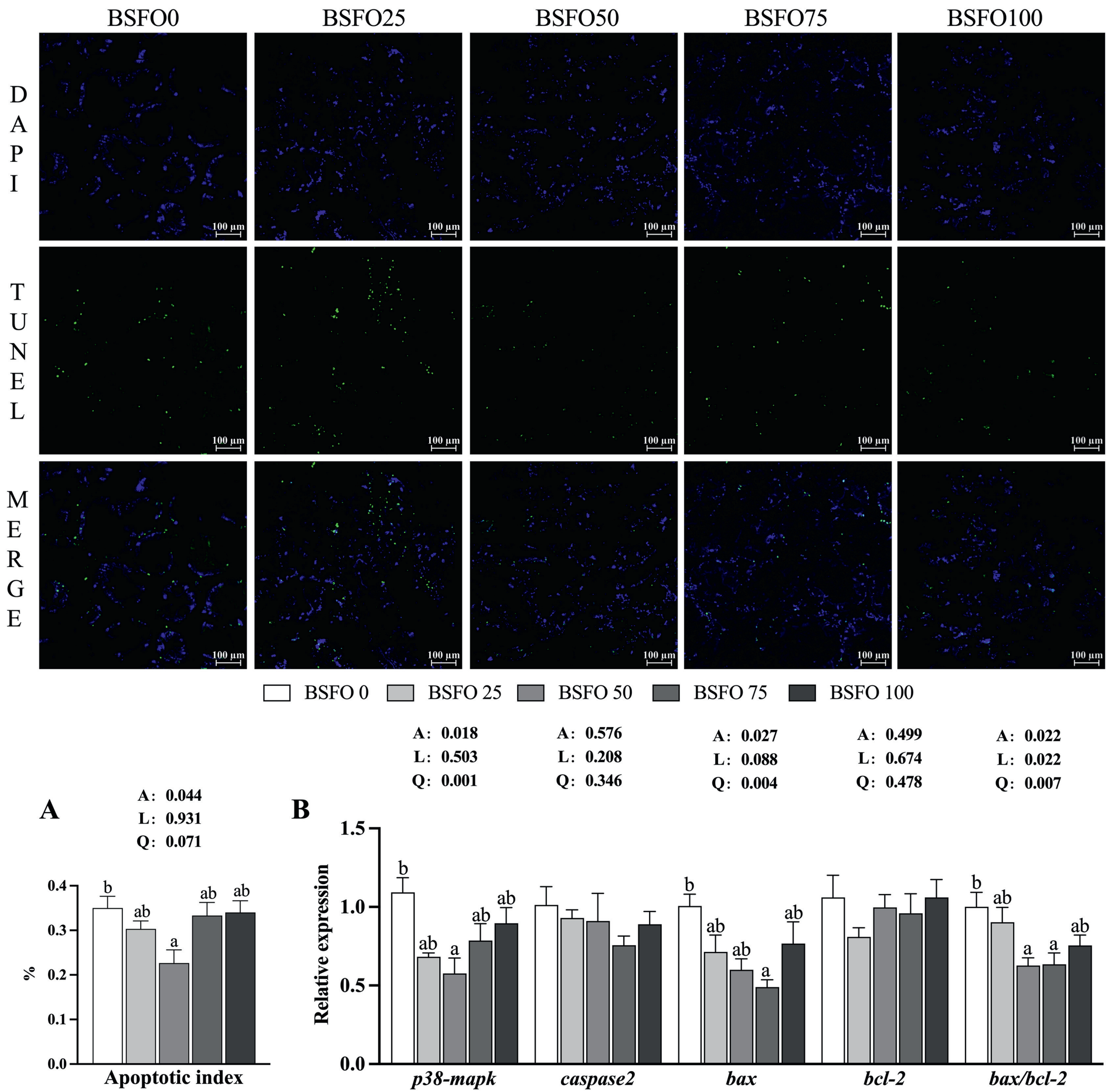

Fig. 3. Effect of replacement of FO with BSFO on apoptosis of the hepatopancreatic cells of mud crab. (A) Apoptotic index. (B) Apoptosis-related genes. Data are expressed as the means ($n = 3$). *p38-mapk* = p38 mitogen-activated protein kinases; *caspase2* = cysteinyl aspartate specific proteinase 2; *bax* = bcl-2 associated x protein; *bcl-2* = B cell lymphoma-2; *bax/bcl-2* = *bax/bcl-2* ratio. [a, b] Mean values with unlike letters are significantly different ($P < 0.05$). FO = fish oil; BSFO = black soldier fly larval oil; A = the variance analyzed by one-way ANOVA; L = linear trend analyzed by orthogonal polynomial contrasts; Q = quadratic trend analyzed by orthogonal polynomial contrasts.

3.7. Expression levels of genes related to antioxidant

As shown in Fig. 4, the expression level of thioredoxin (*trx*) in the hepatopancreas significantly up-regulated with the increase in replacement of FO by BSFO ($P = 0.036$), however, the expression levels of cytoplasmic manganese superoxide dismutase (*cytMn-sod pre*) ($P = 0.243$) and catalase (*cat*) ($P = 0.869$) were not significantly influenced by dietary replacement of FO with BSFO. Crabs fed diets with 0 and 25% replacement of FO with BSFO exhibited higher expression levels of glutathione peroxidase (*gpx*) than those fed the other diets ($P < 0.001$).

3.8. Hepatopancreas histology

As presented in Fig. 5, hepatopancreatic H&E sections and lipid content showed the impact of substituting FO with BSFO on lipid deposition in the hepatopancreas. Crabs fed the diet with 50% replacement of FO with BSFO showed higher R-cell height than those fed the control diet ($P = 0.001$).

3.9. Lipid metabolism

As presented in Fig. 6, crabs fed the control diet had lower expression levels of genes involved in lipid synthesis such as acetyl-

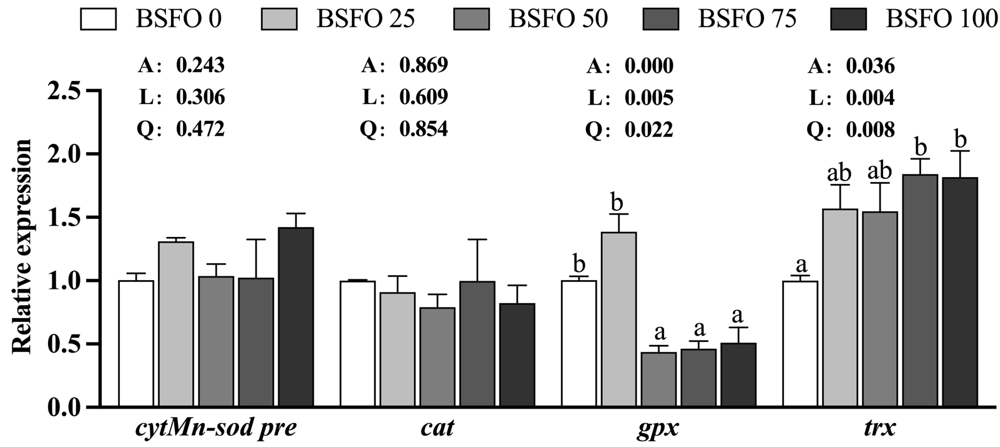

Fig. 4. Effects of replacement of FO with BSFO on the expression levels of genes related to antioxidants in the hepatopancreas of juvenile mud crab. Data are expressed as the means ($n = 3$). [a, b] Mean values with unlike letters are significantly different ($P < 0.05$). *cytMn-sod pre* = cytoplasm manganese superoxide dismutase; *cat* = catalase; *gpx* = glutathione peroxidase; *trx* = thioredoxin. FO = fish oil; BSFO = black soldier fly larval oil; A = the variance analyzed by one-way ANOVA; L = linear trend analyzed by orthogonal polynomial contrasts; Q = quadratic trend analyzed by orthogonal polynomial contrasts.

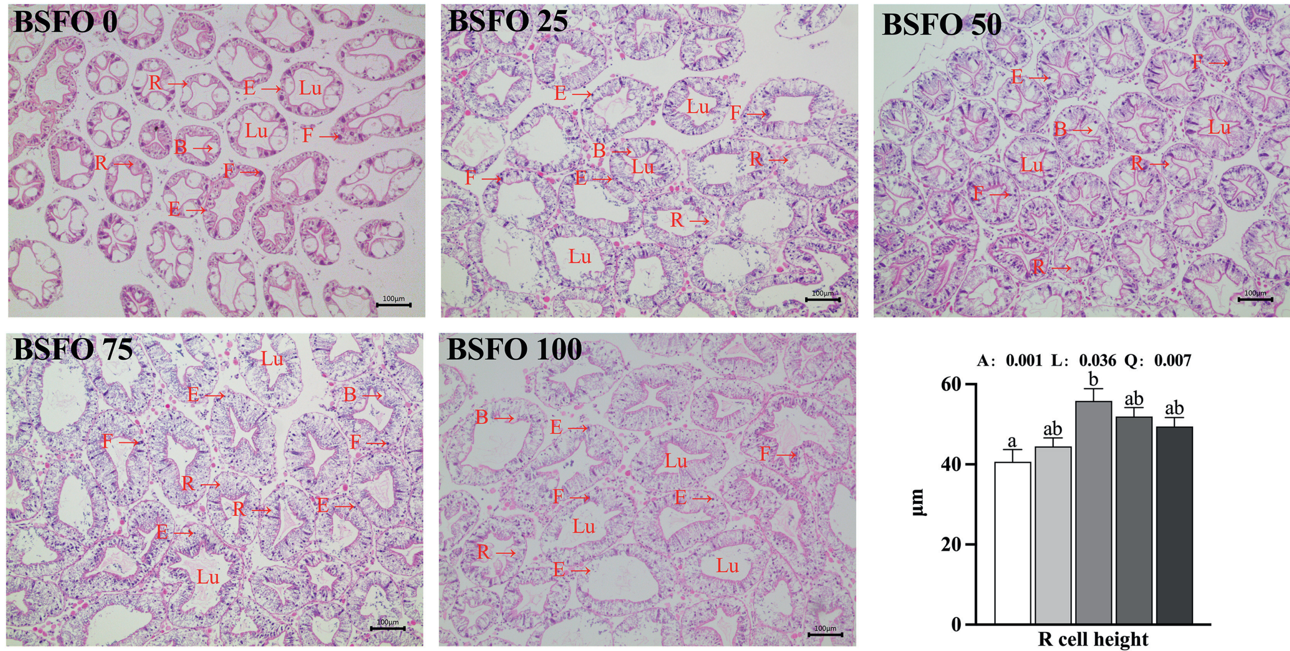

Fig. 5. Effects of replacement of FO with BSFO on the hepatopancreatic histology of juvenile mud crab under magnification 100×. Notes in pictures: F = fibrillar cell; B = blister-like cell; R = resorptive cell; E = embryoid cell; Lu = tubule lumen. R cell height, height of resorptive cell ($n = 9$). Data are expressed as the means ($n = 3$). [a, b] Mean values with unlike letters are significantly different ($P < 0.05$). FO = fish oil; BSFO = black soldier fly larval oil; A = the variance analyzed by one-way ANOVA; L = linear trend analyzed by orthogonal polynomial contrasts; Q = quadratic trend analyzed by orthogonal polynomial contrasts.

CoA (*acc*) and fatty acid synthase (*fas*) in the hepatopancreas than those fed the other diets ($P = 0.016$ and $P = 0.043$). Conversely, the expression levels of genes associated with lipolysis such as carnitine palmitoyltransferase 1 (*cpt1*), acyl-CoA oxidase-1 (*aco1*), and acyl-CoA oxidase-3 (*aco3*) were significantly down-regulated with an increase in dietary replacement of FO with BSFO ($P < 0.05$). The transcript level of genes related to LC-PUFA biosynthesis, such as Δ6 fatty acyl desaturase-like (*Δ6 fad*) was significantly up-regulated with an increase in dietary replacement of FO with BSFO ($P < 0.001$), and crabs fed diet with 50% replacement of FO with BSFO exhibited higher expression levels of Δ9 fatty acyl desaturase-like (*Δ9 fad*) and elongation of very long-chain fatty acid protein 6 (*elovl6*) than those fed the control diet ($P = 0.030$; $P = 0.040$). The lowest expression levels of fatty acid binding protein-1 (*fabp1*) and fatty acid transport protein-4 (*fatp4*) were observed in crabs fed a diet with 50% replacement of FO with BSFO ($P < 0.001$; $P = 0.020$). Meanwhile, in contrast to crabs fed diets with 25% and 100% replacement of FO with BSFO, crabs fed diet with 50% replacement of FO with BSFO showed significantly higher FAS activity in the hepatopancreas (Table 9, $P = 0.021$). Additionally, CPT activity decreased significantly with the increase in the replacement of FO with BSFO ($P = 0.005$). The TG in hepatopancreas significantly increased with dietary replacement of FO with BSFO up to the level of 50% ($P = 0.011$). Moreover, the concentration of TCHO in the hepatopancreas was not significantly affected by dietary replacement of FO with BSFO ($P = 0.096$).

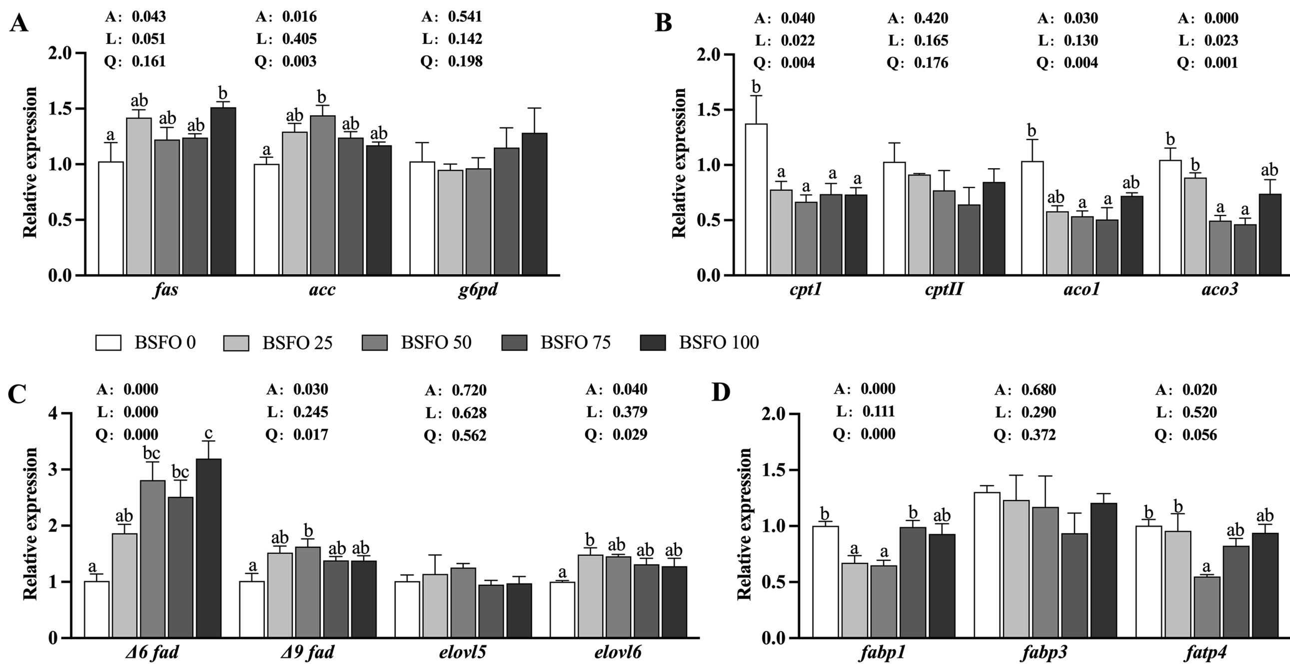

Fig. 6. Effects of replacement of FO with BSFO on the expression levels of genes related to lipid metabolism in the hepatopancreas of juvenile mud crab. (A) Lipid synthesis, (B) lipolysis, (C) long-chain poly unsaturated fatty acids (LC-PUFA) biosynthesis, (D) lipid transport. Data are expressed as the means ($n = 3$). *fas* = fatty acid synthase; *acc* = acetyl-CoA; *g6pd* = glucose 6-phosphate dehydrogenase; *cptI* = carnitine palmitoyltransferase 1; *cptII* = carnitine palmitoyltransferase 2; *aco1* = acyl-CoA oxidase-1; *aco3* = acyl-CoA oxidase-3; *Δ6 fad* = Δ6 fatty acyl desaturase-like; *Δ9 fad* = Δ9 fatty acyl desaturase-like; *elovl5* = elongation of very long-chain fatty acid protein 5; *elovl6* = elongation of very long-chain fatty acid protein 6; *fabp1* = fatty acid binding protein-1; *fabp3* = fatty acid binding protein-3; *fatp4* = fatty acid transport protein-4. [a-c] Mean values with unlike letters are significantly different ($P < 0.05$). FO = fish oil; BSFO = black soldier fly larval oil; A = the variance analyzed by one-way ANOVA; L = linear trend analyzed by orthogonal polynomial contrasts; Q = quadratic trend analyzed by orthogonal polynomial contrasts.

Table 9
Lipid metabolism indices of the hepatopancreas of juvenile mud crabs fed different experimental diets for 8 weeks.

Item	Replacement of FO with BSFO					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
TG, μmol/g	6.40^{a}	9.36^{ab}	11.45^{b}	11.36^{b}	12.51^{b}	0.691	0.011	0.001	0.001
TCHO, μmol/g	2.13	2.15	2.20	2.33	2.63	0.069	0.096	0.009	0.014
FAS, U/g prot	8.45^{ab}	8.27^{a}	10.76^{b}	9.78^{ab}	8.31^{a}	0.328	0.021	0.614	0.065
CPT, U/g prot	12.85^{b}	11.78^{ab}	11.89^{ab}	11.74^{ab}	10.37^{a}	0.245	0.005	0.001	0.004

FO = fish oil; BSFO = black soldier fly larval oil; TG = triglyceride; TCHO = total cholesterol; FAS = fatty acid synthetase; CPT = carnitine palmitoyltransferase.
Data are expressed as the means ($n = 3$).
[a,b] Values with different letters in the same row were significantly different ($P < 0.05$).

3.10. Mitochondrial function

The transmission electron microscope image of the hepatopancreas was presented in Fig. 7. Crabs fed diet with 50% replacement of FO with BSFO exhibited higher mitochondria in the hepatopancreas than those fed the other diet ($P < 0.001$). The activity of succinate dehydrogenase (SDH) in the hepatopancreas significantly increased with dietary replacement of FO with BSFO up to a level of 50%, and then significantly decreased with further increase (Table 10, $P < 0.001$). The highest ATP content in the hepatopancreas occurred in the crabs fed diet with 25% replacement of FO with BSFO ($P = 0.003$). The expression levels of sirtuin-1 (*sirt1*) and sirtuin-3 (*sirt3*) in the hepatopancreas were significantly up-regulated with an increase of dietary replacement of FO with BSFO, and crabs fed the diet with 100% replacement of FO with BSFO showed the highest expression levels of *sirt1* ($P = 0.010$) and *sirt3* ($P < 0.001$) among all treatments. The highest expression level of cytochrome *b* (*cytb*) was observed in crabs fed diet with 50% replacement of FO with BSFO ($P = 0.010$). There was no significant difference in the expression levels of nuclear respiratory factor-1 (*nrf1*) among all treatments ($P = 0.096$).

4. Discussion

Previous studies on BSFO as a lipid source in aquatic feed mainly focused on fish, and there were no studies on crustaceans such as crabs and shrimps. Insects, which are already a component of fish's natural diet and have a low ecological impact and land use need, would be a viable choice (Henry et al., 2015). In recent years, research on the use of insect ingredients as substitutes for fish meal or FO in aqua-feeds has sprung up, and some encouraging findings have prompted experts to conduct more research (Dumas et al., 2018; Fawole et al., 2021; Katya et al., 2017; Moutinho et al., 2021; Hu et al., 2023). The proximate composition of BSFL in different media was different. It has been reported that the crude protein content of the black soldier fly larvae is about 37% to 63%, the lipid content is about 7% to 39%, the main fatty acids are lauric acid (C12:0), palmitic acid and oleic acid (Barragan-Fonseca et al., 2017; Leong et al., 2016). In the present study, growth performance and feed utilization were negatively correlated with dietary replacement of FO with BSFO. There were no significant differences in growth performance and feed utilization in crabs fed diets with 0, 25% and 50% replacement of FO with BSFO, and 75% and 100%

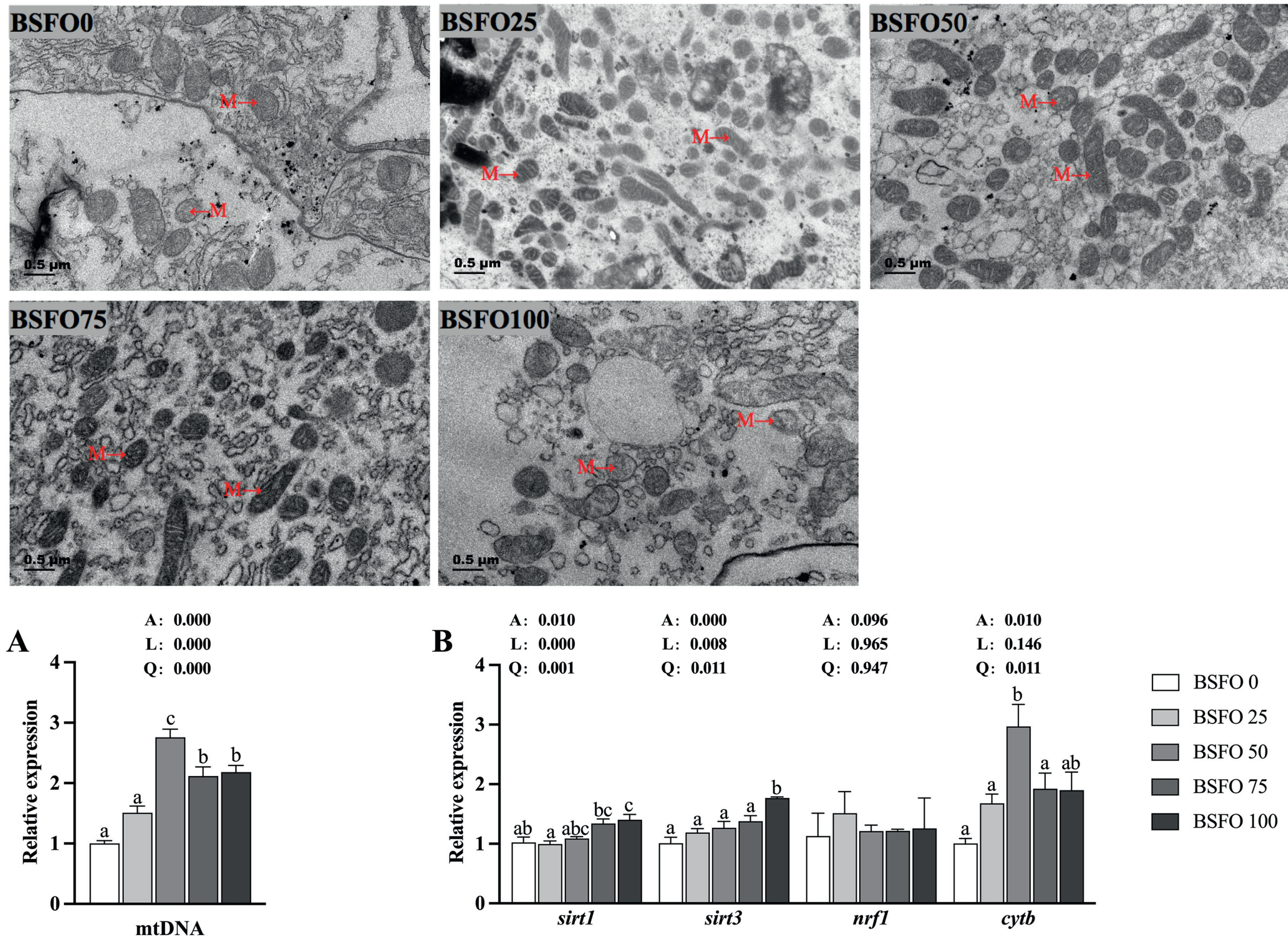

Fig. 7. Effect of replacement of FO with BSFO on the hepatopancreas ultrastructure (magnification 25,000×) and mitochondrial function in juvenile mud crab. Data are expressed as the means (n = 3). M = mitochondrial. (A) Mitochondrial DNA copy number, (B) Energy metabolism-related gene expression. *sirt1* = sirtuin-1; *sirt3* = sirtuin-3; *nrf1* = nuclear respiratory factor-1; *cytb* = cytochrome *b*. [a-c] Mean values with unlike letters are significantly different (P < 0.05). FO = fish oil; BSFO = black soldier fly larval oil; A = the variance analyzed by one-way ANOVA; L = linear trend analyzed by orthogonal polynomial contrasts; Q = quadratic trend analyzed by orthogonal polynomial contrasts.

Table 10
Energy metabolism enzyme activities of the hepatopancreas of juvenile mud crabs fed different experimental diets for 8 weeks.

Item	Replacement of FO with BSFO					SEM	*P*-value		
	0	25%	50%	75%	100%		ANOVA	Linear	Quadratic
SDH, U/mg prot	20.07^{b}	21.06^{b}	27.51^{c}	15.56^{ab}	13.72^{a}	1.385	0.000	0.059	0.008
ATP, μg/mg prot	80.69^{ab}	93.52^{b}	77.22^{ab}	77.52^{a}	74.49^{a}	2.268	0.003	0.004	0.002

FO = fish oil; BSFO = black soldier fly larval oil; SDH = succinate dehydrogenase; ATP = ATP content.
Data are expressed as the means (n = 3).
[a-c] Values with different letters in the same row were significantly different (P < 0.05).

replacement of FO with BSFO significantly inhibited the growth of juvenile mud crabs. The results of the present study are similar to those of previous studies (Abu Bakar et al., 2021; Facey et al., 2023). Previous studies have demonstrated that meeting essential fatty acid requirements with a lipid source that replaces FO does not negatively affect growth performance (Aksakal et al., 2023; Montero et al., 2010; Peng et al., 2017). It can be inferred that the 50% replacement of FO with BSFO in this study will not exceed the tolerance limit for essential fatty acid requirements of mud crabs. In addition, this study only conducted feeding experiments on the juvenile stage of mud crabs, and whether the replacement of FO by BSFO in the diet will have an effect on adult mud crabs remains to be further studied. Moreover, only the crabs fed the diet with 100% replacement of FO with BSFO had significantly lower survival and FE than those fed the control diet, which agreed with the previous study (Agbohessou et al., 2021). However, in the study of replacing FO with BSFO, different studies have reached different conclusions in different fish. For example, rainbow trout could consume 10% BSFO without significantly affecting their growth or ability to absorb and deposit nutrients (Dumas et al., 2018). In the feed of early juvenile *Totoaba macdonaldi*, BSFO can successfully replace 30% of FO without negative effects on growth (Maldonado-Othón et al., 2022). Vegetable oils in the diet of juvenile gilthead seabream could be completely replaced by BSFO (Moutinho et al., 2023). These differences could be attributed to the various aquatic animal species, size, feed formulation, culture conditions, and other lipid supplies. Furthermore, the amount of fish meal added to the feed is also an important factor in determining the replacement level, after

all, fish meal provides about 10% of the FO. Lack of essential fatty acids in the diet or fatty acid imbalances can frequently lead to slower growth and higher death (NRC, 2011). The growth performance and survival in this study were within reasonable limits from published data on similar species (Holme et al., 2007; Huang et al., 2024; Wang et al., 2024). According to the growth results of marine crabs (mud crab) and freshwater crabs (Chinese mitten crab), feeding trials starting from an initial body weight of 5 to 20 g showed that the weight gain rate was 100% to 150%, rarely exceeding 200%, and the survival was mostly in the range of 60% to 80%, which may be determined by the cultured species itself (Zhao et al., 2015; Xu et al., 2020a; Chen et al., 2024). For example, the percent weight gain of a swimming crab with an initial weight of 5 g may reach 700% after 8 weeks of feeding (Wang et al., 2020), but the percent weight gain of a Chinese mitten crab or mud crab with an initial weight of 5 g was less than 150% after 8 weeks of feeding (Li et al., 2023; Peng et al., 2025). In this study, the initial weight of mud crabs is relatively large, and the percent weight gain of crabs is mainly dependent on molting. The larger the crab, the longer the molting cycle and the slower the growth.

The results of this study suggested that the hepatopancreas lipid content of mud crabs fed diets with more than 50% replacement of FO with BSFO was higher than those fed the other diets. On the other hand, the protein content in the hepatopancreas significantly decreased with an increase in dietary replacement of FO with BSFO. The quality of adipose tissue depends on the number and size of lipid cells, which are represented by changes in the size of lipid cells (Moody et al., 2019). Since the resorptive (R) cells are the primary location for lipid storage, they are regarded as a vital sign of the nutritional status of crustaceans (Zarantoniello et al., 2023). In the present study, when the proportion of BSFO replacing FO exceeded 50%, lipid deposition in hepatopancreas was significantly increased, possibly due to a large increase in R cells, which could also be verified by an increase in concentration of TCHO and TG in the hepatopancreas. When MCFA are used in low doses, it decreases TG secretion, and when used in excess, it may increase lipid regeneration. This, in turn, increases the secretion of TG. Since TCHO and TG secretion are synergistically regulated, an increase in TG secretion may lead to a simultaneous increase in TCHO secretion, thereby increasing TCHO levels (Marten et al., 2006). The results of the present study were in agreement with a previous study on juvenile Jian carp (Li et al., 2016). When the BSFO replacement level changed in the range of 0 to 50%, crude lipid content in the liver of juvenile Jian carp tended to increase. In this research, dietary BSFO supplementation induced lipid deposition in the hepatopancreas, which may be the result of up-regulation of lipogenesis and down-regulation of lipolysis, as dietary BSFO supplementation down-regulated the expression levels of genes related to lipolysis and up-regulated the expression of lipogenic-related genes. However, dietary replacement of FO with BSFO had no effect on the contents of protein or lipid in the muscle, which was consistent with previous studies on rainbow trout (Fawole et al., 2021), mirror carp (Xu et al., 2020b) and gilthead seabream (Moutinho et al., 2023).

Fish oil is obtained by processing marine fish. Marine fish generally feed on algae, shrimp, and fish rich in unsaturated fatty acids, and the n-3 PUFA content is rich, especially DHA and EPA. However, the BFL mainly feeds on food waste, especially kitchen waste. Recently, in order to produce stable larvae, some enterprises used plant and animal processing by-products as a medium, which contain a higher level of lauric acid, but a lower level of total fatty acid than marine fish. At the same time, the fatty acid composition of the BSFO changed greatly due to the large differences in the culture medium of the BFL. The acids lauric (C12:0), palmitic (C16:0), and oleic (C18:1n-9) are abundant in BSFO (Surendra et al., 2016). The main fatty acids of BSFO used in the present study were C18:1n-9 (40.62%), followed by C16:0 (23.00%) and C12:0 (13.12%). Fatty acid composition in animal tissues was positively correlated with fatty acid composition in diet (Fountoulaki et al., 2009). In this study, the content of C12:0 in the hepatopancreas of mud crabs dramatically increased with an increase in dietary replacement of FO with BSFO, and the highest contents of total SFA, MUFA, and n-6 PUFA in the hepatopancreas were observed in the crabs fed diet with 100% replacement of FO with BSFO.

Critical metabolic pathways influence not only the absorption of LC-PUFA from food but also the lipid content of tissues, biosynthesis is essential in order to provide an endogenous supply of these crucial functional essential fatty acids (Monroig and Kabeya, 2018). The liver or hepatopancreas is a crucial metabolic organ whose lipid metabolism is controlled by multiple routes, including free fatty acid intake, lipid de novo synthesis, and fatty acid degradation (Liu et al., 2018). In this investigation, the expression levels of lipid synthesis-related genes (*fac*, *acc*) were up-regulated and lipolysis-related genes (*cpt1*, *aco1*, and *aco3*) were down-regulated in the hepatopancreas of mud crabs fed diets with BSFO supplementation. Meanwhile, the expression levels of *elovl6*, *Δ6 fad*, and *Δ9 fad* were up-regulated in the hepatopancreas, and similar results were reported on rainbow trout (Fawole et al., 2021). These results indicated that low levels of n-3 and n-6 polyunsaturated fatty acids in diets activated the synthesis of LC-PUFA, which is consistent with previous studies (Bell et al., 2001; Carvalho et al., 2018).

Lauric acid, one of the MCFA, does not need to be transported across the membrane bilayer through the CPT-1 transporter. It can quickly pass through the membrane bilayer by non-ionic passive diffusion and directly enter the mitochondria to provide energy. Therefore, the large amount of lauric acid in tissues is more suitable for energy than long-chain fatty acids (Dayrit, 2014). As the BSFO contained abundant saturated fatty acid (SFA), they have a certain “n-3 saving effect”. The increase in the proportion of SFA in the feed reduced the metabolism of n-3 LC-PUFA, resulting in increased deposition of these fatty acids in the fillets (Turchini et al., 2011). This was further demonstrated by the down-regulation of *fabp1* and *fatp4* gene expressions in the hepatopancreas in crabs fed diets with BSFO supplementation. Fatty acid binding proteins (FABP) transport and store fatty acids in mitochondria and are membrane proteins expressed in tissues with fatty acid metabolic activity, efficiently promoting the transport of long-chain fatty acids (Acharya et al., 2023). Their down-regulated expression indicates that BSFO reduced the oxidative decomposition of LC-PUFA. These may be the main reasons for fatty acid deposition in the hepatopancreas of mud crabs fed diets with BSFO supplementation. Previous studies have confirmed that a portion of ingested lauric acid enters the bloodstream for rapid metabolism, and only a small portion remains in the liver in the form of triglycerides. The small amount of lauric acid that enters muscles may be used for rapid energy supply rather than accumulation (Debois et al., 2009). The results of this study demonstrated that there are variations in the rise and fall of fatty acid levels between the hepatopancreas and muscle. Obviously, the fatty acid profiles in the hepatopancreas and muscle are not only influenced by diets but also due to the regulation of fatty acid metabolism in crabs. This may be the reason that mud crabs improve the utilization efficiency of BSFO feed through the difference in lipid nutrient metabolism between the hepatopancreas and muscle (Zhou et al., 2023).

Alanine transaminase (ALT) and aspartate aminotransferase (AST) are key enzymes that reflect the health of the liver or hepatopancreas (Nayak et al., 2023). In this research, crabs fed diets with 0 and 25% replacement of FO with BSFO exhibited lower ALT activity in the hemolymph than those fed the other diets, but there was not a significant variance in AST activity among all treatments.

This may be due to the addition of high levels of BSFO in the feed, leading to minor damage caused by lipid deposition in the hepatopancreas. Previous studies have shown that antioxidant capacity is crucial for maintaining good health (Hawkins et al., 2022). Catalase (CAT), superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) make up the majority of the antioxidant enzyme system in animals, which functions to scavenge free radicals and protect cells from damage. As a result, these antioxidant enzymes are frequently used as biomarkers of oxidative stress and as indicators to evaluate the antioxidant status of aquatic species (Wangkahart et al., 2022). In this study, different antioxidant systems responded differently to the addition of dietary BSFO. Crabs fed diets with 25% and 50% replacement of FO with BSFO showed higher concentrations of GSH and GSH-Px and activities of T-SOD, T-AOC, and CAT in the hepatopancreas and hemolymph than those fed the control diet. Meanwhile, the expression levels of genes related to antioxidants, such as *gpx*, reached their highest level in the 25% replacement group, and the expression level of *trx* was significantly up-regulated with an increase in dietary replacement of FO with BSFO. Additionally, as the percentage of BSFO increased, the concentrations of MDA and PC in the hepatopancreas decreased dramatically. It is well known that protein carbonyl (PC) is a valuable marker of protein oxidation and a product of protein oxidation, which makes it an indicator of oxidative stress (Spencer et al., 2022). Malondialdehyde (MDA) is a lipid peroxidation product that serves as a typical marker of impaired antioxidant capacity (Thanpari et al., 2015). Simultaneously, mitochondria are abundant in antioxidant actives that are crucial for eliminating reactive oxygen species and maintaining the body's redox balance (Salimi et al., 2021). In the present study, the number of hepatopancreas mitochondria increased in the 25% and 50% FO replacements, further confirming that BSFO enhanced the antioxidant capacity of mud crabs. In conclusion, replacing the appropriate level of FO with BSFO in the diet has a greater effect on improving the antioxidant capacity of mud crabs, which is consistent with the results of previous studies.

Nitric oxide synthase (NOS) and nitric oxide (NO) are key molecules in the antimicrobial defense produced by the innate immune system of crustaceans (Rodriguez-Ramos et al., 2016). Lysozyme (LZM) is a membrane-bound organelle responsible for invading and destroying pathogens that enter the host cells (Jin et al., 2023). The activity of phenoloxidase (PO) is the most significant and well-studied indicator for monitoring the immune response of crustaceans (Tanner et al., 2006). In this study, the crabs fed a diet with 50% replacement of FO with BSFO showed higher activities of immune-related enzymes (NO, NOS, LZM and PO) in the hemolymph and hepatopancreas than those fed the other diets. The results suggested that optimal dietary replacement of FO with BSFO enhanced the immunity of mud crab, which is consistent with the results of other investigations (Chen et al., 2022; Cho et al., 2022; Foysal et al., 2021). Chitin and lauric acid in BSF larvae may be the main reasons for the enhancement of non-specific immunity (Chia et al., 2020). Both chitin and lauric acid have been shown to have immune properties (Siddaiah et al., 2023). Recent findings suggested that bioactive peptides contained in insects can inhibit processes that induce inflammation in fish (Xiang et al., 2019). These peptides have antibacterial properties, but they may also have particular innate immunomodulatory effects (Nogales-Mérida et al., 2018). The effect of BSFL meal or oil on the antioxidant and immune capacity of fish and crustaceans remains to be further studied in the future.

TUNEL results also confirmed the above findings, as the increase in the proportion of BSFO replacing FO in the diet decreased the apoptosis signal of hepatopancreas cells and reached the lowest level in the crabs fed diet with 50% replacement of FO with BSFO. Apoptosis is a cellular self-protection mechanism that maintains body homeostasis by eliminating excess, damaged, or potentially harmful cells (Ming et al., 2014) and is primarily controlled by apoptosis-related proteins (BAX, CASPASE, BCL-2, JNK, BOK and P38-MAPK). BCL-2 is a key regulator of intrinsic apoptosis and protects cells from death, whereas BAX is a major activator of the apoptotic pathway (Gu et al., 2017). In this research, the expression levels of *p38-mapk* and *bax* in the hepatopancreas of mud crabs fed diets with 50% and 75% replacement of FO with BSFO were significantly down-regulated and reached the lowest level, respectively. BAX/BCL-2 ratio > 1 suggested that apoptosis was increased (Salakou et al., 2007). The results of this study demonstrated that the BAX/BCL-2 ratio was significantly down-regulated (<1) in the BSFO groups. Therefore, it is speculated that the dietary BSFO supplementation reduced apoptosis by enhancing the antioxidant and immune capacities of mud crabs.

Dietary lipids are essential for metabolism, especially energy metabolism, where they provide energy for growth, turnover, and maintenance of homeostasis processes (Luo et al., 2023). Mitochondria are the main source of energy generation in eukaryotic cells and play a key role in cell growth, differentiation, cell signaling, apoptosis, and cell cycle control (Bratic and Trifunovic, 2010). Perry et al. (2015) found that enhanced mitochondrial function protects against excessive lipid accumulation, oxidative stress, and inflammation. Thus, antioxidant capability and lipid metabolism are intimately linked to mitochondria. Quinone substrate binding and transmembrane electron transfer, which convert redox energy into a proton motive force, are both carried out by Cytb. The cell metabolism sensor Sirt1 monitors the energy status of cells and controls cell physiology and energy requirements in response to nutrient uptake, such as fatty acid (Luo et al., 2023). Sirt3 deacetylates and activates a variety of target substrates for fatty acid oxidation, essential for regulating mitochondrial energy metabolism and activating ATP synthesis (Yuan et al., 2019). The labeling enzyme SDH is found in mitochondria and is crucial to the tricarboxylic acid cycle and oxidative phosphorylation, which boosts ATP generation (Ardehali et al., 2004; Cao et al., 2023). In this research, the mitochondria of the hepatopancreas in each group had a clear morphology and neat ridge arrangement. In addition, dietary 25% and 50% replacement of FO with BSFO increased the number of mitochondria in the hepatopancreas of crabs, elevated SDH activity, and increased mitochondrial DNA copy numbers. The highest content of ATP occurred in the crabs fed diet with 25% replacement of FO with BSFO, and the results suggested that an appropriate BSFO replacement level can enhance the mitochondrial energy metabolism of mud crabs. The expression levels of *sirt1*, *sirt3*, and *cytb* in the hepatopancreas of the crab were significantly up-regulated when fed diets with BSFO supplementation, which further confirmed that the dietary BSFO supplementation improved mitochondrial function in the mud crab. Based on these findings, it may be concluded that dietary BSFO supplementation can improve mitochondrial function, thereby improving lipid metabolism and antioxidant performance in mud crabs.

5. Conclusions

In conclusion, substituting 25% to 50% FO with BSFO (i.e. in-feed rate of 0.75% to 1.5% BSFO) had no negative effects on growth performance, feed efficiency, or fatty acid composition in the hepatopancreas and muscle of juvenile mud crabs. Moreover, dietary replacement of FO with BSFO could improve the antioxidant capacity and immune response, thereby alleviating hepatopancreas cell apoptosis. Furthermore, dietary BSFO may promote mitochondrial biosynthesis and energy metabolism homeostasis in the

hepatopancreas by regulating the lipid metabolism of juvenile mud crabs. However, it is not clear how the dietary BSFO supplementation affects adult mud crabs, and future investigations should assess growth performance and physiological and metabolic responses throughout the growing period.

CRediT authorship contribution statement

Yuhang Yang: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Tingting Zhu:** Methodology, Formal analysis. **Min Jin:** Writing – review & editing, Conceptualization. **Xiangkai Li:** Resources, Formal analysis. **Shichao Xie:** Methodology, Formal analysis, Data curation. **Yuhui Cui:** Formal analysis. **Qicun Zhou:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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 A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2025.01.002.

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