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Original Research Article

Aquafeed fermentation improves dietary nutritional quality and benefits feeding behavior, meat flavor, and intestinal microbiota of Chinese mitten crab (*Eriocheir sinensis*)

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ABSTRACT

Normally, proper fermentation can be an efficient and widely used method to improve feed quality in animal rearing; however, the studies on crustaceans, especially *Eriocheir sinensis*, remain limited. This study aimed to investigate whether feed fermentation could meliorate dietary nutritional value and benefit *E. sinensis* rearing. First, non-fermented feed (NFD) and fermented feed (FD) were produced and assessed, respectively. Then, the "Y" maze feed choice behavior test (180 times; 30 times, 6 rounds) was conducted to assess the attractiveness of these 2 feeds for crabs. Finally, a total of 80 crabs (44.10 ± 0.80 g) were randomly assigned into 2 groups with 4 replicates, and fed the experimental diets for 8 weeks to evaluate the effects of each feed on growth, antioxidant capacity, meat flavor, and intestinal microbiota. In this study, FD showed higher levels of crude protein ($P < 0.01$), soluble protein ($P < 0.01$), amino acids ($P < 0.05$), lactic acid ($P < 0.001$), and lower levels of crude fiber ($P < 0.05$) and antinutritional factors (agglutinin, trypsin inhibitor, glycinin, and β-conglycinin) ($P < 0.001$) than NFD. Additionally, FD was more attractive to crabs than NFD ($P < 0.01$) and it stimulated the appetite of crabs more than NFD ($P < 0.05$). The growth performance, feed efficiency, and digestive enzyme activity of FD-fed crabs were significantly higher than those of NFD-fed crabs ($P < 0.05$). The electronic sensory measurements and free amino acid profiles revealed that the FD diet had positive impacts on the meat flavor of crabs, particularly in "sweet" and "umami" tastes. Moreover, the antioxidant capacity of FD-fed crabs was significantly higher than that of NFD-fed crabs ($P < 0.05$). Fermented feed also affected the diversity and composition of intestinal microflora. The functional prediction of microbial communities showed that crabs fed FD had a better microecological environment in the intestine. In conclusion, the fermentation of aquafeed could be an effective approach to enhance feed quality and therefore benefit *E. sinensis* rearing.

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1. Introduction

Feedstuff is an important component in the aquaculture industry, with its cost accounting for approximately 70% of total production inputs (Dawood and Koshio, 2019). The price of feed ingredients, especially fish meal, has been progressively rising in recent years, which has resulted in researchers exploring alternatives to cope with the rising cost (Heinzl et al., 2022). Using plant protein sources as much as possible (such as soybean meal, cottonseed meal, peanut meal, and rapeseed meal) to reduce fish meal in aquafeed has been seen as a practical way to reduce costs.

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Although plant proteins are relatively widely available and have much lower prices, their usability is limited due to poor palatability, high fiber content, low protein content, amino acid imbalance, and various antinutritional factors (ANFs) (Francis et al., 2001; Gatlin et al., 2007). As a result, excessive use of plant protein sources induces enteritis, dysbacteriosis, oxidative stress, and immune disorders in fish (Francis et al., 2001; Gu et al., 2016; Wang et al., 2016). Currently, solid-state fermentation is considered one of the most promising schemes for improving the quality of feedstuff (Dawood and Koshio, 2019). Fermentation was found to be effective in decomposing ANFs, crude fiber, and carbohydrates, improving the content of crude protein and peptides, and ameliorating the amino acid pattern in plant sources (Liu et al., 2017; Shi et al., 2017b; Wang et al., 2019b; Bueno et al., 2020). Apart from improving nutritional value, the fermentation process was also reported to increase the production of antioxidants in the feed like γ-aminobutyric acid, phenolics, flavonoids, etc. (Yang et al., 2021).

The main bacterial strains used for fermentation include *Bacillus*, *Enterococcus*, *Lactobacillus*, *Saccharomyces*, and *Aspergillus*, and using different microorganisms in the fermentation process leads to different effects (Dawood and Koshio, 2019; Yang et al., 2021). *Bacillus* and *Aspergillus* are the main strains used for the fermentation of protein because they have strong protein and carbohydrate-digesting enzyme-secretion abilities (Suprayogi et al., 2022). Chi and Cho (2016) reported that *Bacillus subtilis* dramatically improved the absorption, digestion, and bioactivity of soybean meal in fermentation. *Enterococcus* and *Lactobacillus* are commonly used for carbohydrate fermentation, converting carbohydrates into lactic acid, and assisting in the breakdown of some proteins and fibers (Graham et al., 2020). *Lactobacillus* plays an important role in the sensory, nutritional, and hygiene of various fermented products, and produces a variety of aromatic compounds that impart flavor (Pogačić et al., 2015; Zhang and Vadlani, 2015). *Saccharomyces* (yeast) is a eukaryotic single-cell microorganism that can convert carbohydrates into carbon dioxide and alcohol (Yang et al., 2021), and is often used in the production of biomass, since it can rapidly utilize a variety of carbon sources and produce a large amount of protein (Kurcz et al., 2018). Dworschack and Wickerham (1961) documented that, regardless of the carbon source, *Saccharomyces cerevisiae* and other yeasts were generally inferior to *Candida utilis* in the production of extracellular and total invertase, as well as lactic acid, glycerol, and ethyl alcohol. Moreover, recent studies have shown that the use of mixed strains under suitable conditions, allowing the advantages of each strain to be utilized, could achieve better fermentation effects than a single strain (Dawood and Koshio, 2019; Yang et al., 2021). Hence, strains including *B. subtilis*, *C. utilis*, and *Lactobacillus plantarum* subsp. *plantarum* were chosen in this study for producing a kind of representative fermented aquafeed.

In terms of animal nutrition, fermented feed (FD) has been successfully used for a long time with very positive outcomes (Dawood and Koshio, 2019; Yang et al., 2021). Numerous studies documented that FD improves growth performance (Semjon et al., 2020; Zhang et al., 2021), feed efficiency (Hassaan et al., 2018; Semjon et al., 2020), digestibility (Wang et al., 2014; Zhang et al., 2021), immunity (Li et al., 2020a; Zhu et al., 2020), antioxidant capacity (Semjon et al., 2020; Zhang et al., 2021), and intestinal microbiota (Li et al., 2020a; Zhang et al., 2021) of cultured animals. Interestingly, FD also impacts the meat quality of aquatics. Tanaka et al. (2016) found that the concentrations of free amino acids, which are related to good taste (i.e., umami and sweet), were increased in the carp muscle after feeding with thermophile-fermented compost. Jannathulla et al. (2019) also reported significant changes in eicosatetraenoic acid (C20:5) and docosahexaenoic acid (C22:6) contents of shrimp fed with different levels of fermented soybean meal. Studies on aquatic animals have mainly focused on substituting fish meal with fermented products, yet there have limited studies on the effects of fermented compound feed.

The Chinese mitten crab, *Eriocheir sinensis*, is a traditional food in China and a symbol of Chinese cuisine. Crab meat is popular due to its unique and desirable flavor, which can be referred to as “sweet” and “umami” tastes (Chen and Zhang, 2007). Over recent decades, the crab has become an essential commercial freshwater aquaculture species. The scale of its farming has risen steadily and the annual production has reached nearly 800,000 tonne according to Bureau of Fisheries of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2020. Several features of this special species have been studied during long-term artificial culturing. Because of its weak neural and humoral regulation, *E. sinensis*, as a primitive crustacean, is vulnerable to various environmental stressors produced by the rapid development of intensive aquaculture (Ishii et al., 2013). In addition, although the gastrointestinal tract of a crab is composed of a foregut (mouth, esophagus, and stomach), midgut, and hindgut, its digestion and absorption capacity are probably limited with a relatively simple straight tube (Babu et al., 1982). Meanwhile, crabs are slow in foraging and eating, which may lead to feed waste and water pollution (Hartati and Briggs, 1993). Findings from previous studies on crustaceans such as Pacific white shrimp (Li et al., 2020b; Zhang et al., 2021) have provided some insight into how FD affects crab cultivation in terms of growth, digestibility, immunity, and antioxidant capacity. However, whether FD has positive effects on the feeding behavior and meat flavor of *E. sinensis* remains unknown. Moreover, there is also limited documentation available on the intestinal microbiota of *E. sinensis* fed microbial-fermented diets.

In response to the points mentioned above, this study aimed to investigate the effects of fermentation on the nutritional quality of crab compound feed and determine whether the fermented feed could exhibit favorable impacts on feed foraging, meat flavor, and intestinal flora in *E. sinensis* rearing. Thus, the nutritional value of compound feed fermented with *B. subtilis*, *C. utilis*, and *L. plantarum* subsp. *plantarum* was assessed, after which a feed choice behavior experiment was performed. Furthermore, the bioavailability of FD to crabs was determined after an 8-week feeding trial.

2. Materials and methods

2.1. Animal ethics statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Nanjing Agricultural University (NAU), Nanjing, China. The experiments on *E. sinensis* complied with the ARRIVE guidelines (Percie du Sert et al., 2020).

2.2. Microorganisms and culture conditions

Microorganisms used in this study conformed to the established specification of the *2013 Catalog of Feed Additives* (announcement No. 2045 of the Ministry of Agriculture and Rural Affairs, China). *B. subtilis* (CICC 10071), *C. utilis* (CICC 1314), and *L. plantarum* subsp. *plantarum* (CICC 6009) were obtained from the China Center of Industrial Culture Collection (Beijing, China).

Microorganisms were cultured under specific conditions (medium, temperature, and duration) according to the supplier’s instructions. *B. subtilis* was inoculated into an Erlenmeyer flask containing peptone, 10.0 g; beef extract, 10.0 g; glucose, 10.0 g; sodium chloride (NaCl), 5.0 g; distilled water, 1.0 L, and incubated at 30 °C for 24 h with shaking. *C. utilis* was inoculated into an Erlenmeyer flask containing 5° Bé malt-extract, 1.0 L, and incubated at

30 °C for 72 h with shaking. *L. plantarum* subsp. *plantarum* was inoculated into an Erlenmeyer flask containing peptone, 10.0 g; beef extract, 10.0 g; yeast powder, 5.0 g; glucose, 5.0 g; CH_3COONa, 5.0 g; $C_6H_{14}N_2O_7$, 2.0 g; Tween 80, 1.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCO_3$, 20.0 g; distilled water, 1.0 L, and incubated under static and anaerobic conditions at 30 °C for 48 h.

2.3. Fermentation procedure and feed preparation

The formula of the basal compound feed in this study was designed for *E. sinensis* (Table 1). For the preparation of non-fermented feed (NFD), ingredients were filtered through an 80-mesh sieve and then thoroughly mixed before adding lipid sources. Water was added last to comprise 30% of the feed weight, and the mixture was extruded and pelleted through a 2.0-mm diameter die. The feed was completely dried at 60 °C for 24 h, and stored at −20 °C in sealed plastic bags until use.

Table 1
Ingredients of basal compound feed (dry matter basis).

Ingredients	Content, %
Fish meal [1]	32.00
Peanut meal [2]	12.00
Corn [3]	10.00
Soybean meal [4]	6.40
Blood powder [5]	6.00
Wheat bran [6]	3.60
Cottonseed meal [7]	3.60
Alpha-starch [8]	18.00
Fish oil [9]	2.00
Soy oil [10]	2.00
Monocalcium phosphate [11]	1.50
Attapulgite [12]	1.00
Premix [13]	1.00
Cholesterol [14]	0.10
Lecithin [15]	0.15
Choline chloride [16]	0.10
Ethoxyquin [17]	0.05
Sodium chloride [18]	0.50
Total	100.00

[1] Fish meal: from Superprime, TASA Fish Product Co., Ltd., Peru.
[2] Peanut meal: from Hipore Feed Co., Ltd., Taizhou, China.
[3] Corn: from Hipore Feed Co., Ltd., Taizhou, China.
[4] Soybean meal: from Dahai Oils& Grains Industrial (Fangchenggang) Co., Ltd., China.
[5] Blood powder: from Jianrun Biotechnology Co., Ltd, Lianyungang, China.
[6] Wheat bran: from Yihai Kerry (Zhengzhou) Food Industry Co., Ltd., China.
[7] Cottonseed meal: from Yihai (Changji) Oils& Grains Industrial Co., Ltd., China.
[8] α-starch: from Henan Hengrui Starch Technology Co., Ltd., Luohe, China.
[9] Fish oil: from Coland Feed Co., Ltd., Wuhan, Hubei, China.
[10] Soybean oil: from Yihai Kerry Arawana Holdings Co., Ltd., Shanghai, China.
[11] Monocalcium phosphate: from Guizhou Chuanheng Chemical Co., Ltd., Fuquan, China.
[12] Attapulgite: from Huamu Animal Science and Technology Institute Co., Ltd, Nanjing, China.
[13] Premix supplied the following minerals and vitamins (per kilogram diet): $CuSO_4 \cdot 5H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 250 mg; $ZnSO_4 \cdot 7H_2O$, 220 mg; $MnSO_4 \cdot 4H_2O$, 70 mg; Na_2SeO_3, 0.4 mg; KI, 0.26 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; vitamin A, 9,000 IU; vitamin D, 2,000 IU; vitamin E, 45 mg; vitamin K_3, 2.2 mg; vitamin B_1, 3.2 mg; vitamin B_2, 10.9 mg; vitamin B_5, 20 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.016 mg; vitamin C, 100 mg; pantothenate, 10 mg; folic acid, 1.65 mg; choline, 600 mg; biotin, 1 mg; myoinositol, 150 mg.
[14] Cholesterol: from Ruixiang Biotechnology Co., Ltd., Shanghai, China.
[15] Lecithin: from Ruixiang Biotechnology Co., Ltd., Shanghai, China.
[16] Choline chloride: from Huamu Animal Science and Technology Institute Co., Ltd., Nanjing, China.
[17] Ethoxyquin: from Huamu Animal Science and Technology Institute Co., Ltd., Nanjing, China.
[18] Sodium chloride: from China National Salt Industry Co., Ltd., Huaian, China.

For the preparation of the FD, the conditions used in analogous studies (Shi et al., 2017b; Bao et al., 2018; Zhang et al., 2021) and the growth requirements of the microorganisms were considered and tested in preliminary experiments before the fermentation procedure was finalized. The final fermentation procedure was as follows: first, 100.0 g of NFD ingredients (moisture approximately 40%) were placed in a 500 mL Erlenmeyer flask and covered with polytetrafluoroethylene-vented sealing film (Biosharp Life Sciences Co., Hefei, China); next, the NFD was inoculated with *B. subtilis* (8.0 log CFU/g) and fermented at 30 °C for 24 h with regular shaking to provide enough oxygen. Afterwards, the Erlenmeyer flask was inoculated with *C. utilis* (8.0 log CFU/g) and *L. plantarum* subsp. *plantarum* (8.0 log CFU/g) and then placed into an anaerobic culture bag containing an anaerobic gas-producing bag (Hopebio Biotechnology Co., Qingdao, China). The mixture was fermented at 30 °C for 60 h, then extruded and pelleted through a 2.0-mm diameter die immediately. The feed was completely dried at 60 °C for 24 h, and stored at −20 °C in sealed plastic bags until use.

The entire feed-producing and fermentation experiment was repeated to obtain enough feed for the animal trials, and samples of NFD and FD were collected in triplicate and stored at −80 °C for further analysis.

2.4. Feed choice behavior experiment

To compare the crabs' preference for NFD or FD, the "Y" maze feed choice behavior experimental device was used, based on the design of Bool et al. (2011) with minor modifications. Feed choice behavior tests were carried out 180 times in total (30 times, 6 replicates) with a single randomly selected, starved crab each time. Each crab was tested only once.

First, the water outlet was closed, and the 2 water inlets were connected to the same water source. The device was filled with purified water up to 50 cm in depth. The inlet and outlet water quantities were adjusted to maintain the total volume of water in the device as a constant with a slow and steady flow. Thereafter, a crab was placed in the area separated by a division plate with holes. Once the crab was stable, the same weights of NFD and FD were provided at a fixed position near the water inlets of the device, before the partition plate was opened. Feed was replaced for every replicate, and the position of the NFD and FD were exchanged. The number of times each feed was selected by the crab was recorded.

During the procedure, the stability and quietness of the experimental environment, device, and personnel remained unchanged. The results were analyzed by the Wilcoxon-Mann-Whitney two-sample test.

2.5. Animal rearing and sample collection

Experimental crabs were obtained from the Fisheries Teaching and Research Base of Nanjing Agricultural University (Nanjing, China). Crabs, with an average weight of 44.10 ± 0.80 g, were reared in a recirculation system (1.0 m × 1.0 m × 1.0 m, length × width × height) containing fabric-filtered and aerated city water (0.8 m^3). Disinfected polyvinyl chloride tubes were provided as shelters and crabs were acclimated in this environment for 1 week before the beginning of the experiment. Crabs were fed a commercial feed (Hipore Feed Co., Taizhou, China), of which the proximate composition was as follows: moisture, 10.0%; crude protein, 38.0%; crude lipid, 6.5%; ash, 16.0%; and crude fiber, 6.5%.

Only those crabs in the intermolt period were chosen for the experiment.

At the beginning of the feeding experiment, 80 crabs were randomly assigned to 2 groups with 4 replicates ($n = 10$ per replicate). The 2 experimental groups were the NFD group (fed with NFD), and the FD group (fed with FD). A daily ration of about 2.5% body weight (estimated by observing feed intake situation) was split into 2 meals fed at 08:00 and 18:00 to each pool for 8 weeks. The actual feeding rate (amount) was adjusted every 2 weeks by reweighing crabs. During the cultivation period, water quality parameters were measured and adjusted regularly. Temperature, dissolved oxygen, and pH of water, detected using a multiparameter SevenGo Pro-SG6 (METTLER TOLEDO Measurement Technology Ltd., Zurich, Switzerland), were kept at 26.0 ± 2.0 °C, 6.5 ± 0.5 mg/L, and 7.9 ± 0.2, respectively; and unionized ammonia was maintained at < 0.05 mg/L (Model Analyzer Typeranges AN300, Teledyne Technologies Incorporated, San Diego, United States).

Crabs in each replicate were collected to get a total weight, and 3 crabs that weighed close to the average weight were sampled after the feeding experiment. Hemolymph was collected using syringes, in which it was mixed with the same volume of precooling anticoagulant solution (100 mmol/L glucose, 26 mmol/L citrate, 30 mmol/L citric acid, 450 mmol/L NaCl, 10 mmol/L EDTA, and pH 7.2). The mixture was immediately centrifuged (1,200 × *g*, 4 °C, 20 min) to obtain the supernatant, which was stored at −80 °C. Then crabs were anesthetized in ice for 15 min before dissecting. The hepatopancreas and intestine were dissected aseptically and frozen immediately in liquid nitrogen, then stored at −80 °C for subsequent analysis. Meat (mixed from claws, legs, and abdomen) was manually separated on ice and stored at −80 °C before further use.

2.6. Chemical and biochemical analysis

2.6.1. Molecular weight distribution of proteins

To intuitively demonstrate the changes in dietary proteins after fermentation, the molecular weight distribution of proteins in experimental feed was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The feed samples were thoroughly ground, and 0.1 g was homogenized with 2.0 mL of 20 mmol/L Tris−HCl buffer containing 0.1% SDS, 5 mmol/L dithiothreitol, and 5 μg/mL protease inhibitor and left on ice for 1 h. The homogenized mixtures were centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatants were transferred into new tubes. The protein concentration in each sample was determined using the BCA protein assay kit (Beyotime Biotechnology Co., Shanghai, China) and adjusted to 100 μg/mL. The SDS-PAGE system employed a 12% polyacrylamide for the separating gel and 4% for the stacking gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 solution and de-stained with a methanol-acetic acid solution to visualize the protein bands.

2.6.2. Proximate composition, ANFs, and lactic acid content

NFD and FD were analyzed for moisture, crude protein, crude lipid, ash, and crude fiber using the AOAC official methods of analysis (AOAC, 1997). The water-soluble protein and acid-soluble protein content of samples were determined using the methods described by Bueno et al. (2020) and Chen et al. (2010), respectively. Glycinin, β-conglycinin, agglutinin, and trypsin inhibitor contents were analyzed using enzyme-linked immunosorbent assay kits (MM-1614O1, MM-2304O1, MM-0131O1, and MM-1613O1, respectively; MEIMIAN Bio Co., Yancheng, China) according to the manufacturer's protocol. The lactic acid (PubChem CID: 612) concentration was determined by high-performance liquid chromatography as described by Bao et al. (2018).

2.6.3. Hydrolyzed amino acids and free amino acids

The L-8900 automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan), equipped with a guard column (5.0 mm × 4.0 mm, i.d. 5 μm) and an analytical column (60 mm × 4.6 mm, i.d. 3 μm) packed with Hitachi custom ion exchange resin, was used to determine the amino acid profiles of feeds and the free amino acid concentrations in crab meats.

To determine the amino acid content of the feed, 50.0 mg feed was weighed and hydrolyzed in a test tube containing 5.0 mL of 6 mol/L HCl. Air was removed from the test tube using a vacuum pump, and the tube was airproofed with an alcohol blowtorch. The feed was then placed into a thermal container. Hydrolysis was maintained at 110 °C for 24 h. The test tube was removed and opened after cooling; the diluted sample was transferred to a 50.0-mL capacity bottle and adjusted to 50.0 mL. A dilution (10.0 mL) was transferred to a distillation flask in a water bath at 60 °C and evaporated using a rotary evaporator (RE-52AA, Yarong Biochemical Instrument Co., Ltd, Shanghai, China). The residue was dissolved in 10.0 mL 0.02 mol/L HCl and 1.0 mL of the mixture was filtered through a 0.20 μm syringe filter (SLLGX13, Millipore Co., Billerica, United States) for testing.

To measure the concentration of free amino acids in crab meat, 3.0000 g of meat sample was weighed into a triangular flask with a plug. Then 15.0 mL of 10.0% sulfosalicylic acid solution was added, and the flask was placed in a boiling water bath for 5 min to obtain the supernatant. The residue was extracted 3 times according to the above steps. All the supernatants were transferred to a 50.0-mL volumetric flask, and the volume was adjusted with pH 2.2 sodium citrate buffer. The supernatant (1.0 mL) was filtered through a 0.20-μm syringe filter (SLLGX13, Millipore Co., Billerica, United States) for testing.

2.6.4. Antioxidant indicators

Hemolymph samples were used to analyze antioxidant capacity. The content of protein carbonyl (PCO) was measured using an assay kit purchased from Solarbio Biotechnology Co. (BC1275, Beijing, China), which produces red-colored 2,4-dinitrophenylhydrazone with a characteristic absorption peak at 370 nm from the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine. The content of malondialdehyde (MDA), the enzyme activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as the total antioxidant capacity (T-AOC), were determined using commercial kits purchased from Jiancheng Bioengineering Institute (A003-1-2, A001-3-2, A007-1-1, A005-1-2, and A015-2-1, respectively; Nanjing, China). The content of MDA was detected by the thiobarbituric acid (TBA) method. The enzyme activities of SOD and CAT were measured using the 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium Sodium Salt (WST-8) method, and the ammonium molybdate method, respectively. The GPx assay relies on the oxidation of glutathione (GSH) and reduction of H_2O_2 through the catalytic activity of GPx, resulting in the production of oxidized glutathione (GSSG); glutathione reductase (GR) reduces GSSG to lower GSH using nicotinamide adenine dinucleotide phosphate (NADPH), which has a characteristic absorption peak at 340 nm. T-AOC was determined by the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Ammonium Salt (ABTS) method.

2.6.5. Digestive enzyme activities

The homogenate of hepatopancreas samples stored at −80 °C was used for digestive enzyme activities analysis. Protease activity was evaluated according to Classics Lowry et al. (1951) using casein

as the substrate and reacting it with Folin phenol reagent. Amylase activity was measured according to Pan and Wang (1997) using soluble starch as the substrate with the 3, 5-dinitrosalicylic acid (DNS) method. Lipase activity was measured according to the method from Duncombe (1963) based on the content of blue chromium complex due to the reaction between the fatty acids and the copper ions.

2.7. Electronic sensory evaluation

2.7.1. Electronic nose

Differences in the odor of crab meat samples were measured using a FOX α4000 Sensory Array Fingerprint Analyzer (Alpha MOS Co., Toulouse, France) equipped with 18 types of metal oxide array sensors. These sensors were used to detect specific flavor substances, and the model number of each sensor and the substances that can be identified are listed in Table 2. To perform the experiment, 5.0 g of raw meat sample was placed in a glass tube with a plug and heated in boiled water for 20 min, which produces cooked meat while reducing the loss of flavor substances. Then 5.0 mL of 0.18 g/mL NaCl solution was added and the mixture was homogenized using a T10 basic ULTRA-TURRAX homogenizer (IKA-Werke GmbH Co., Staufen, Germany) at a speed of 20,000 rpm for 60 s (an ice-water mixture was used for cooling). Approximately 2.0 g of the mixture was put in a 10-mL headspace bottle for testing. The headspace was maintained at 60 °C for 600 s before injection. The carrier gas was clean air and its flow rate was 150 mL/min. The temperature of the injection module was 60 °C; the injection speed was 2,500 μL/s; the injection period was 1 s. The data acquisition time was 120 s and the detection time was 100 s. After each sample detection, the sensors were cleaned with clean air for 1200 s.

2.7.2. Electronic tongue

Differences in the taste of crab flesh were measured using an ASTREE potentiometric electronic tongue (Alpha MOS Co., Toulouse, France) equipped with 7 chemical-selective sensors (AHS, PKS, CTS, NMS, CPS, ANS, and SCS) and 1 reference electrode (Ag/AgCl). The AHS, CTS, NMS, ANS, and SCS sensors detect sour, salty, umami, sweet, and bitter tastes, respectively. The other 2 sensors, PKS and CPS, are auxiliary sensors that detect integrated signals. To perform the experiments, 10.0 g of raw meat sample was placed in a glass tube with a plug and heated in boiled water for 20 min. After adding 100 mL of distilled water, the mixture was homogenized using a T10 basic ULTRA-TURRAX homogenizer (IKA-Werke GmbH Co., Staufen, Germany) at a speed of 20,000 rpm for 60 s (an ice-water mixture was used for cooling). The mixture was centrifuged (10,000 × *g*, 15 min, 4 °C) and the supernatant was filtered through a 0.20 μm syringe filter (SLLGX13, Millipore Co., Billerica, United States). The filtered liquid was used for analysis after calibration and testing of the sensors. The data acquisition frequency of the sensors was once per second, and the data acquisition period of each sample was 120 s (i.e. the data of 120 tests was collected). The data collected at 120 s was used for final analysis because sensor signals reached their most stable state at this time.

Table 2
Electric nose sensors and detected substances.

Number	Sensor	Detected substances
1	LY2/LG	Chlorine, fluorine, nitrogen oxides, sulfide
2	LY2/G	Ammonia, amine compounds, carbon oxides
3	LY2/AA	Ethanol, acetone, ammonia
4	LY2/GH	Ammonia, amine compounds
5	LY2/gCT1	Hydrogen sulfide
6	LY2/gCT	Propane, butane
7	T30/1	Polar compounds, hydrogen chloride
8	P10/1	Nonpolar compounds, hydrocarbons, ammonia, chlorine
9	P10/2	Nonpolar compounds, methane, ethane
10	P40/1	Fluorine, chlorine
11	T70/2	Toluene, xylene, carbon monoxide
12	PA/2	Ethanol, ammonia, amine compounds
13	P30/1	Hydrocarbons, ammonia, ethanol
14	P40/2	Chlorine, hydrogen sulfide, fluoride
15	P30/2	Hydrogen sulfide, ketone
16	T40/2	Chlorine
17	T40/1	Fluorine
18	TA/2	Ethanol

2.8. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA from the hepatopancreas was extracted using TRIzol reagent (Vazyme Biotech Co., Nanjing, China). RNA concentration and purity were determined using an ND-1000 UV Spectrophotometer (Nano Drop Technologies Inc., Wilmington, United States). Synthesis of cDNA was conducted using a reverse transcription kit (Vazyme Biotech Co., Nanjing, China); cDNA was then amplified using an SYBR Green real-time PCR Mix kit (Vazyme Biotech Co., Nanjing, China) on a QuantStudio 5 Real-Time PCR machine (Applied Biosystems Co., Glenville, United States). The reaction parameters were as follows: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s; the melt curve was recorded at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. S27 was selected as the reference gene (Huang et al., 2017). The CDS sequences of cholecystokinin (*CCK*) and leptin (*LEP*) were obtained from our laboratory's database of the hepatopancreas transcriptome sequences of *E. sinensis*. All primers were designed online (NCBI, United States) (Table 3) and results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Intestinal microbiota analysis

The intestine-retained content samples obtained from crabs were sent to Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) for microbial community detection. Amplification and sequencing of the V4–V5 region of the bacterial 16S DNA gene were performed using barcoded fusion primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The raw reads were produced by Flash software (Version 1.2.11) (Magoč and Salzberg, 2011). Sequences were compared with the SILVA database (Version 138) (Quast et al., 2012) using Qiime software (Version 1.9.1) (Caporaso et al., 2010) to detect and remove chimera sequences. Then, the clean reads were obtained and clustered into Operational Taxonomic Units (OTUs) with ≥97% similarity by using Uparse software (Version 11) (Edgar, 2013). The representative sequence for each OTU was screened for further annotation. For each representative sequence, the SILVA Database was used based on Mothur (Version 1.30.2) (Haas et al., 2011) algorithm to annotate taxonomic information. Alpha and beta diversity analyses were also performed using Qiime software. Furthermore, the potential function of intestinal microbiota was analyzed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, Version 1.1.0).

2.10. Statistical analysis

Statistical analysis was performed using SPSS software (Version 22.0, IBM Co. Ltd., Armonk, United States). $P < 0.05$ was used to indicate a statistically significant difference. The Wilcoxon-Mann-Whitney two-sample test was used in the feed choice behavior

Table 3
Primer sequences used for RT-qPCR.

Gene	Primer sequence (5′–3′)	Primer length, bp	Product length, bp	Reference/GenBank no.
S27	F: GGTCGATGACAATGGCAAGA	20	105	Huang et al. (2017)
	R: CCACAGTACTGGCGGTCAAA	20		
CCK	F: GCCTCATCCCCTACATGCAA	20	98	Transcriptome Sequences
	R: AAGCGGTTGACAGATAGCGT	20		
LEP	F: TGGCATACTTCGTGACTGCC	20	180	
	R: CGTCGTCACTGTCGGCTATC	20		
SOD	F: TAAACTGGCGATTGCGACCT	20	273	FJ617306.1
	R: GCCAAGGCATTAGCAAGTGG	20		
CAT	F: GCTATGGCTCCCACACCTTT	20	148	GU361618.1
	R: CGGATGGCATAGTCAGGGTC	20		
GPx	F: TTGCTGACCAAGATCGGGAC	20	267	FJ617305.1
	R: ATGGCACCACCATACACTGG	20		
Trx1	F: CAGAAGCTTGTTGTCGTGGA	20	219	FJ372908.1
	R: TGCTCCAGAGAAGCTGTCAA	20		
Prx6	F: CTGGCTGTGACCCTTGGTAT	20	186	EU626070.1
	R: TTTTGTCAGCTGGAGGGAGT	20		

bp = base pairs; F = forward; R = reverse; *S27* = ubiquitin/ribosomal S27 fusion protein; *CCK* = cholecystokinin; *LEP* = leptin; *SOD* = superoxide dismutase; *CAT* = catalase; *GPx* = glutathione peroxidase; *Trx1* = thioredoxin 1; *Prx6* = peroxiredoxin 6.

experiment. All other data were tested for equality of variances with Levene's test and independent sample *t*-tests were used afterwards to analyze significance. The data were expressed as mean ± standard deviation.

3. Results

3.1. Nutritional composition of experimental feeds

The crude protein content of FD was 5.90% higher than that of NFD ($P < 0.01$; Table 4) and the ratio of water-soluble protein to acid-soluble protein in crude protein was 31.13% ($P < 0.01$) and 28.91% ($P < 0.001$; Table 4) higher in FD than in NFD, respectively. The generation of lactic acid was also significantly higher in FD (60.88 mg/g) than in NFD, where it was nearly undetectable ($P < 0.01$; Table 4). In addition, the crude fiber content was lower in FD (5.74%) than in NFD (6.71%; $P < 0.05$; Table 4). No significant differences were observed in crude lipid and ash between the feeds ($P > 0.05$; Table 4).

The amounts of 4 ANFs, including agglutinin, trypsin inhibitor, glycinin, and β-conglycinin were significantly lower in FD than in NFD (all $P < 0.001$; Table 4). Their degradation rates (change from NFD to FD) reached 92.28%, 72.96%, 79.93%, and 84.19%, respectively.

The proportions of 5 essential amino acids (Arg, Ile, Met, Phe, Thr) and 3 non-essential amino acids (Asp, Ser, Gly) were significantly higher in FD than in NFD ($P < 0.05$; Table 4). Notably, the content of total non-essential amino acids and total amino acids was 16.52% and 10.96% higher in FD than in NFD, respectively ($P < 0.05$; Table 4). The proportion of the other 9 amino acids (His, Leu, Lys, Val, Glu, Ala, Cys, Tyr, Pro), as well as total essential amino acids, were not significantly different between feeds ($P > 0.05$; Table 4).

The molecular weight distributions of proteins in NFD and FD were presented by SDS-PAGE. Most high molecular weight proteins in the NFD were metabolized into proteins of lower molecular weight by fermentation (as marked with the red rectangle, Fig. 1).

3.2. Choice behavior test of experimental feeds

Across 6 rounds comprising 180 tests in total, NFD was chosen 78 times (13, 10, 16, 12, 14, and 13 times, respectively), whereas FD was chosen 102 times (17, 20, 14, 18, 16, and 17 times, respectively) by crabs (Fig. 2). The feed choice behavior test showed crabs had a significantly higher preference to forage for FD than for NFD ($P < 0.01$).

3.3. Growth performance of crabs fed with experimental feeds

Crabs in the FD group had a significantly higher final weight, weight gain rate, and specific growth rate than those in the NFD group (all $P < 0.05$; Table 5). Compared with the NFD, the FD also resulted in a significantly lower rate of feed conversion and a significantly higher protein efficiency ratio (both $P < 0.05$; Table 5). However, there were no significant differences in feed intake, survival rate, molting time, hepatopancreas index, or gonadosomatic index (all $P > 0.05$; Table 5).

3.4. Antioxidant and digestive-related biochemical indicators of crabs fed with experimental feeds

Activities of the enzymes SOD, CAT, GPx, and T-AOC in the FD group were significantly higher than those in the NFD group ($P < 0.05$; Table 6). By contrast, the oxidative stress markers PCO and MDA in hemolymph were significantly lower in crabs from the FD group than in those from the NFD group (both $P < 0.05$; Table 6).

The enzyme activities of the protease, amylase, and lipase in the hepatopancreas of crabs fed with FD were significantly increased compared with those fed with NFD after the feeding trial ($P = 0.000$, $P = 0.029$, and $P = 0.008$, respectively; Table 6).

3.5. Meat flavor analysis of crabs fed with experimental feeds

3.5.1. Electronic nose analysis

The results of odor analysis of crab meat using an electronic nose were first analyzed by principal component analysis (PCA; Fig. 3 A). The cumulative contribution of the first principal component (PC 1, 90.21%) and the second principal component (PC 2, 4.81%) was 95.02% (>85%). Therefore, the first principal component accounted for a high proportion of variability within the odor samples. There was no overlap between samples from the 2 groups, indicating that PCA analysis effectively distinguished between them.

When results were visualized with a heat map, the signal intensities from 6 sensors (LY2/LG, LY2/G, LY2/AA, LY2/GH, LY2/gCTL, and LY2/gCT) showed minimal differences between feeding groups, but the remaining 12 sensors showed different signals between the NFD and FD groups (Fig. 3B). Based on the substances that these sensors can detect (Table 2), the feeds appeared to differ in polar

Table 4
Proximate composition, antinutritional factors, and amino acids in experimental feeds (dry basis).

Index	NFD	FD	*t*-value	*P*-value
Proximate composition				
Moisture, %	8.84 ± 0.16	9.16 ± 0.16	−2.455	0.070
Crude protein (CP), %	37.31 ± 0.26	39.51 ± 0.57	−6.049	0.004**
Crude lipid, %	6.97 ± 0.19	7.23 ± 0.12	−1.993	0.117
Ash, %	13.22 ± 0.33	13.95 ± 0.81	−1.458	0.219
Crude fiber, %	6.71 ± 0.31	5.74 ± 0.30	3.867	0.018*
Water-soluble protein, % CP	10.28 ± 0.56	13.48 ± 0.53	−7.223	0.002**
Acid-soluble protein, % CP	14.08 ± 0.41	18.15 ± 0.43	−11.902	0.000***
Lactic acid, mg/g	0.17 ± 0.05	60.88 ± 1.35	−77.605	0.000***
Antinutritional factors, mg/g				
Agglutinin	2.46 ± 0.13	0.19 ± 0.02	35.326	0.000***
Trypsin inhibitor	1.96 ± 0.16	0.53 ± 0.02	17.345	0.000***
Glycinin	16.34 ± 1.23	3.28 ± 0.30	17.882	0.000***
β-conglycinin	7.78 ± 0.95	1.23 ± 0.39	11.031	0.000***
Amino acids				
Essential amino acids, g/100 g				
Arginine (Arg)	2.19 ± 0.07	2.48 ± 0.16	−3.003	0.040*
Histidine (His)	0.96 ± 0.01	0.87 ± 0.11	1.451	0.220
Isoleucine (Ile)	1.34 ± 0.03	1.43 ± 0.05	−2.833	0.047*
Leucine (Leu)	2.64 ± 0.08	2.67 ± 0.17	−0.232	0.828
Lysine (Lys)	2.00 ± 0.05	1.84 ± 0.17	1.580	0.189
Methionine (Met)	0.37 ± 0.05	0.48 ± 0.01	−3.609	0.023*
Phenylalanine (Phe)	1.54 ± 0.04	1.70 ± 0.05	−4.647	0.010*
Threonine (Thr)	1.28 ± 0.06	1.44 ± 0.05	−3.593	0.023*
Valine (Val)	1.70 ± 0.05	1.71 ± 0.09	−0.291	0.785
Total essential amino acids	14.03 ± 0.34	14.64 ± 0.50	−1.738	0.157
Non-essential amino acids, g/100 g				
Asparagine (Asp)	3.06 ± 0.11	3.36 ± 0.14	−2.897	0.044*
Serine (Ser)	1.22 ± 0.03	1.42 ± 0.10	−3.463	0.026*
Glutamine (Gln)	4.68 ± 0.13	5.76 ± 0.68	−2.690	0.055
Glycine (Gly)	1.80 ± 0.04	2.10 ± 0.17	−3.031	0.039*
Alanine (Ala)	2.04 ± 0.04	2.07 ± 0.19	−0.268	0.802
Cysteine (Cys)	0.22 ± 0.01	0.22 ± 0.02	0.294	0.783
Tyrosine (Tyr)	0.71 ± 0.09	1.03 ± 0.23	−2.160	0.097
Proline (Pro)	2.98 ± 0.39	3.52 ± 0.20	−2.133	0.100
Total non-essential amino acids	16.71 ± 0.40	19.47 ± 1.02	−4.344	0.012*
Total amino acids	30.74 ± 0.68	34.11 ± 1.16	−4.328	0.012*

NFD = non-fermented feed; FD = fermented feed. Independent sample *t*-tests were used to analyze significance ($P < 0.05$).
The data were expressed as mean ± standard deviation ($n = 3$).
* Correlation was significant at $P < 0.05$.
** Correlation was significant at $P < 0.01$.
*** Correlation was significant at $P < 0.001$.

compounds, nonpolar compounds (hydrocarbons, ammonia, chlorine), aromatics (toluene, xylene), and amines.

3.5.2. Electronic tongue analysis

The results from the taste analysis of crab meat using an electronic tongue were also analyzed by PCA (Fig. 3C). The cumulative contribution of PC 1 (56.14%) and PC 2 (33.52%) was 89.66% (>85%). Therefore, the first 2 PCs accounted for a high proportion of variability within the taste samples. The data points from the 2 feed groups were completely separate, indicating that the taste difference between samples could be distinguished by PCA.

In addition, a radar chart used to visualize the results of crab meat samples analyzed with the electronic tongue indicated that crab meat from the FD group had slightly stronger sweet and umami tastes than that from the NFD group (Fig. 3D).

3.5.3. Analysis of free amino acids

Concentrations of all free amino acids measured in crab meat tended to be higher in the FD group than in the NFD group; for Asp, Ser, Pro, Thr, Val, Tyr, Ile, and Phe, this difference reached statistical significance ($P < 0.05$; Table 7). As a result, total free amino acid content (pleasant, unpleasant, and all) was significantly higher in meat from the FD group than in meat from the NFD group ($P < 0.01$; Table 7). Finally, the taste activity values (TAV) of each free amino acid in crab meat (calculated by the ratio between its concentration determined in the crab meat and its taste threshold value generally measured in water) also tended to be higher in the FD group than in the NFD group (Table 7).

3.6. mRNA expression levels of appetite and antioxidant-related genes

The mRNA expression levels of 2 appetite-suppressing genes (*CCK* and *LEP*) were significantly lower in the FD than in the NFD group ($P < 0.05$; Fig. 4), while 5 antioxidant genes (*SOD*, *CAT*, *GPx*, *Trx1*, and *Prx6*) were significantly higher in crabs from the FD group than those from the NFD group ($P < 0.05$; Fig. 4).

3.7. Analysis of intestinal microbiota

3.7.1. Richness and diversity

Alpha diversity metrics were calculated from the rarefaction curves at the OTUs level for each group. The Shannon, Simpson, and Chao indices obtained for all the samples in groups were reported to assess the alpha diversity of the intestinal microbiota of *E. sinensis* (Table 8). The FD group had significantly higher Shannon ($P = 0.004$) and lower Simpson ($P = 0.011$) indices than the NFD group. Besides, the Chao index showed no significance between 2 groups ($P > 0.05$).

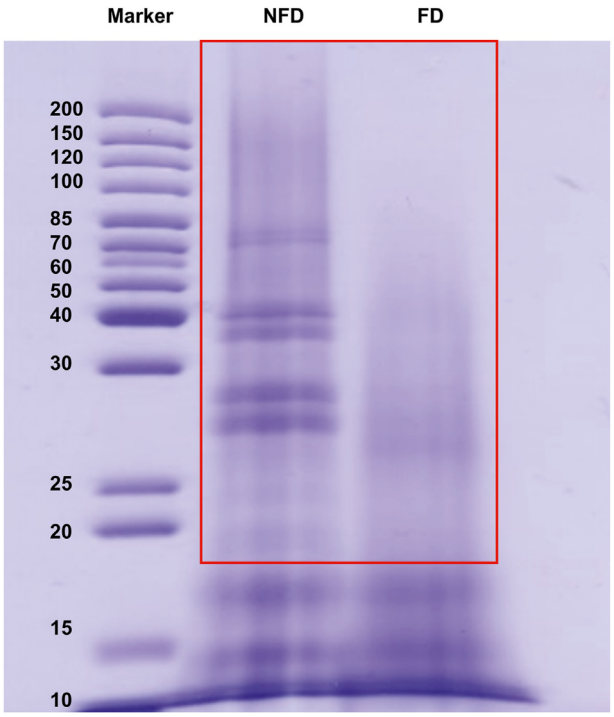

Fig. 1. SDS-PAGE analysis of the 2 experimental feeds. Marker, protein molecular weight markers (10–200 kDa). The red rectangle indicates the degradation protein. NFD = non-fermented feed; FD = fermented feed.

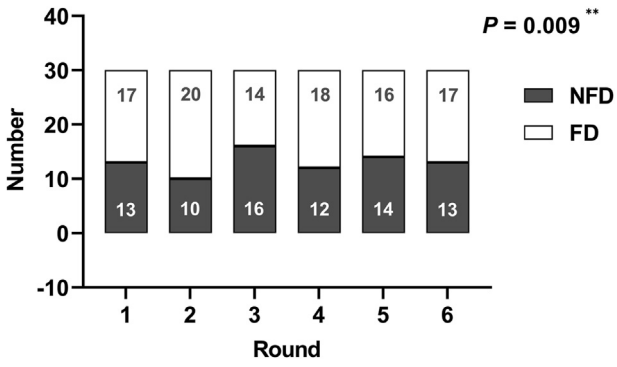

Fig. 2. Number of *Eriocheir sinensis* choosing different feeds in the "Y" maze feed choice behavior test. Data were analyzed by the Wilcoxon-Mann-Whitney two-sample test to detect significant differences between paired samples ($P < 0.05$). ** Correlation was significant at $P < 0.01$. NFD = non-fermented feed; FD = fermented feed.

Beta diversity was demonstrated by principal coordinate analysis (PCoA) and nonmetric multidimensional scaling analysis (NMDS). As presented in Fig. 5, samples of the NFD and FD groups showed no obvious overlaps in both PCoA and NMDS using unweighted UniFrac distances.

A Venn diagram was constructed to identify the core and different OTUs existing in shrimp samples under different diets. In this regard, 200 OTUs were shared among all crab gut samples (Fig. 6). In contrast, 17 OTUs and 19 OTUs were unique to NFD and FD diets, respectively.

3.7.2. Microbiota composition, relative abundance analysis, and comparison

At the phylum level, it was observed that Proteobacteria and Firmicutes were the most abundant phyla. In contrast to the abundance of Proteobacteria, the abundance of Firmicutes was higher in the FD group than that observed in the NFD group (Fig. 7A).

At the class level, the most abundant taxonomic groups for crab gut samples were Gammaproteobacteria and Bacilli. In the NFD group, Gammaproteobacteria was the dominant class, whereas, Bacilli changed to the first place in the FD group. Moreover, Bacteroidia increased obviously in the FD group compared with the NFD group (Fig. 7B).

At the genus level, the microflora compositions (proportion $\geq$0.01%) in the intestine of crabs are represented in Fig. 8A, and those significantly changed are presented additionally in Fig. 8 B. Compared with the NFD group, the FD group had a lower abundance of *Morganella* ($P = 0.01307$), but the abundances of *Candidatus_Hepatoplasma*, *Candidatus_Bacilloplasma*, and *Shewanella* showed the opposite pattern ($P < 0.05$).

3.7.3. Functional prediction

The abundance of functional categories based on KEGG (level 2) between each group was analyzed (Fig. 9). The metagenomics potential of the FD group compared with the NFD group was significantly different in several KEGG pathways. In terms of metabolism function (Fig. 9 E), where more than half of unigenes were mapped, the FD group was significantly enriched in amino acid metabolism, biosynthesis of other secondary metabolites, and metabolism of terpenoids and polyketides compared with the NFD group $P < 0.05$. However, the trends of energy metabolism, and xenobiotics biodegradation and metabolism were reversed ($P < 0.05$). The changing trends of unigenes affiliated with the other 5 functions (KEGG, level 1) were inconsistent as well.

4. Discussion

In this study, we firstly compared the nutritional value of NFD and FD for *E. sinensis*. Similar to our result (Table 4), previous studies reported a higher content of crude protein in fermented feedstuffs (Shi et al., 2017a, 2017b; Wang et al., 2019b). The current understanding postulated that as the content of nitrogen in the fermentation system is fixed, microbial metabolism consumes carbohydrates during the fermentation, resulting in a relative increase in protein content (Mukherjee et al., 2016). Compared with the content of protein, the proportion of crude lipid and ash in feed is relatively low, so there were no significant changes after fermentation in our study; similar results have also been reported by Wang et al. (2019b) and Yao et al. (2018). The content of crude fiber in feed was lower in FD than in NFD in our study (Table 4), which was likely due to the digestion of cellulose by microorganisms capable of generating cellulase during fermentation, for instance, *B. subtilis* (Bi et al., 2015) and *L. plantarum* (Scheirlinck et al., 1990).

In aquaculture, contents of some amino acids in formula diets are insufficient for cultured animals, and exogenous supplementation is a common solution. However, it was reported that crystalline amino acids supplied in the diet were more easily leached out in the water and more rapidly degraded in the gastrointestinal tract than protein-bound amino acids (Williams et al., 2001). Fortunately, feed fermentation through some bacterial and fungal species may overcome these shortages (Anupama and Ravindra, 2000). During the fermentation process, microorganisms can make use of non-protein nitrogen and high molecular weight protein in feedstuffs for growth, reproduction, and synthesis of bacterial proteins with high nutritional value (Wang et al., 2022). In the present study, the general amino acid content of the FD was higher than that of the NFD in our study (Table 4), which was in agreement with previous studies (Bao et al., 2018; Yao et al., 2018; Wang et al., 2019b). However, the content of Lys, one of the most

Table 5
Growth performance of *Eriocheir sinensis* fed with different experimental feeds.

Index	NFD	FD	*t*-value	*P*-value
Initial weight, g	44.70 ± 0.42	44.40 ± 0.59	0.717	0.513
Final weight, g	87.71 ± 2.99	100.41 ± 4.40	−4.129	0.015*
Weight gain rate[1], %	96.23 ± 6.70	126.14 ± 9.92	−4.330	0.012*
Specific growth rate[2], %/day	1.12 ± 0.06	1.35 ± 0.07	−4.380	0.012*
Feed intake[3], g	141.25 ± 11.91	139.03 ± 9.26	1.403	0.233
Feed conversion ratio [4]	3.54 ± 0.49	2.49 ± 0.11	3.614	0.022*
Protein efficiency ratio [5]	0.77 ± 0.10	1.02 ± 0.04	−3.922	0.017*
Survival rate[6], %	75.00 ± 5.77	82.50 ± 5.00	−1.964	0.097
Molting times [7]	14.25 ± 1.26	15.00 ± 0.82	−1.000	0.356
Hepatopancreas index [8]	8.38 ± 0.69	8.47 ± 0.36	−0.368	0.717
Gonadosomatic index [9]	4.98 ± 0.43	4.84 ± 0.44	0.584	0.569

NFD = non-fermented feed; FD = fermented feed.
Independent sample *t*-tests were used to analyze significance ($P < 0.05$). The data were expressed as mean ± standard deviation ($n = 4$). * Correlation was significant at $P < 0.05$.
[1] Weight gain rate (%) = [(average final weight, g) − (average initial weight, g)]/(average initial weight, g) × 100.
[2] Specific growth rate (%/day) = [ln (average final weight, g) − ln (average initial weight, g)]/(56 days) × 100.
[3] Feed intake per crab (g) = ∑ (daily feed intake, g)/(daily survival number).
[4] Feed conversion ratio = (feed intake, g)/[(average final weight, g) − (average initial weight, g)].
[5] Protein efficiency ratio = [(average final weight, g) − (average initial weight, g)]/(protein intake, g).
[6] Survival rate (%) = (final number)/(initial number) × 100.
[7] Molting times = ∑ (daily molt number)/(daily survival number).
[8] Hepatopancreas index = (hepatopancreas weight, g)/(body weight, g).
[9] Gonadosomatic index = (sexual gland weight, g)/(body weight, g).

Table 6
Antioxidant indicators and digestive enzymes of *Eriocheir sinensis* fed with different experimental feeds.

Index	NFD	FD	*t*-value	*P*-value
Antioxidant indicators				
T-AOC, mmol/L Trolox unit	0.50 ± 0.02	0.52 ± 0.01	−2.974	0.011*
SOD, U/mL	11.91 ± 1.25	15.17 ± 1.71	−3.079	0.022*
CAT, U/mL	5.98 ± 0.29	6.68 ± 0.42	−2.714	0.035*
GPx, U/mL	10.42 ± 1.34	13.03 ± 1.48	2.912	0.020*
PCO, nmol/mg prot	0.35 ± 0.05	0.26 ± 0.04	3.205	0.018*
MDA, nmol/mL	6.51 ± 0.48	5.32 ± 0.50	3.400	0.014*
Digestive enzymes				
Protease, U/mg prot	185.58 ± 8.95	294.42 ± 15.16	−12.365	0.000***
Amylase, U/mg prot	1.80 ± 0.06	1.99 ± 0.12	−2.850	0.029*
Lipase, U/g prot	17.52 ± 0.50	19.56 ± 0.93	−3.857	0.008**

NFD = non-fermented feed; FD = fermented feed; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; PCO = protein carbonyl; MDA = malondialdehyde.
Independent sample *t*-tests were used to analyze significance ($P < 0.05$).
The data were expressed as mean ± standard deviation ($n = 4$).
* Correlation was significant at $P < 0.05$.
** Correlation was significant at $P < 0.01$.
*** Correlation was significant at $P < 0.001$.

important limiting amino acids, decreased after fermentation; we also found that the increase of total essential amino acid content was not significant (Table 4). The generation of different amino acids is associated with many factors in fermentation, such as strain, time, substrate, and so on. Park et al. (2010) have pointed out that the content of Lys in *Cheonggukjang* fermentation was related to the fermentation time. Jannathulla et al. (2018) confirmed that a specific strain (*Aspergillus niger*) fermentated under suitable conditions can increase limiting amino acids like Met and Lys. Thus, it was difficult to ensure that all indicators are optimal in fermentation. Non-essential amino acids also play important roles in the life of organisms, and the traditional concept of “ideal protein” has been proved to be more and more closely related to non-essential amino acids nowadays (Wu et al., 2013; Hou and Wu, 2017). Generally, considering the significantly higher levels of non-essential amino acids and total amino acids in the FD, the fermentation in our study indeed benefited the amino acid composition of the feed.

Peptides can be directly absorbed through the animal gut, and are more readily transported and utilized than amino acids (Gilbert et al., 2008). The content of small protein molecules is therefore an important index of FD quality. Previous studies have reported elevated peptide concentrations in FD using water-soluble and acid-soluble proteins as proxy markers (Chen et al., 2010; Bueno et al., 2020). We also found higher levels of both types of protein in the FD than in the NFD (Table 4). In addition, SDS-PAGE is commonly used to separate soluble proteins in feed and visually display the molecular distribution (Shi et al., 2017b; Wang et al., 2019b; Bueno et al., 2020); the bands in our stained gel were generally similar with those published previously (Fig. 1). Furthermore, we detected 4 typical ANFs in plant protein materials (agglutinin, trypsin inhibitor, glycinin, and β-conglycinin), which were degraded after fermentation by 92.28%, 72.96%, 79.93%, and 84.19%, respectively (Table 4). Shi et al. (2017b) found that the degradation rate of glycinin and β-conglycinin was about 80% in corn-soybean meal mixed feed fermented with *B. subtilis* and *Enterococcus faecium*. Moreover, Zhang et al. (2017) reported that solid-state fermentation of soybean meal with *B. subtilis*, *S. cerevisiae*, and *B. lactis* under suitable conditions led to a 95% decomposition rate of protease inhibitor.

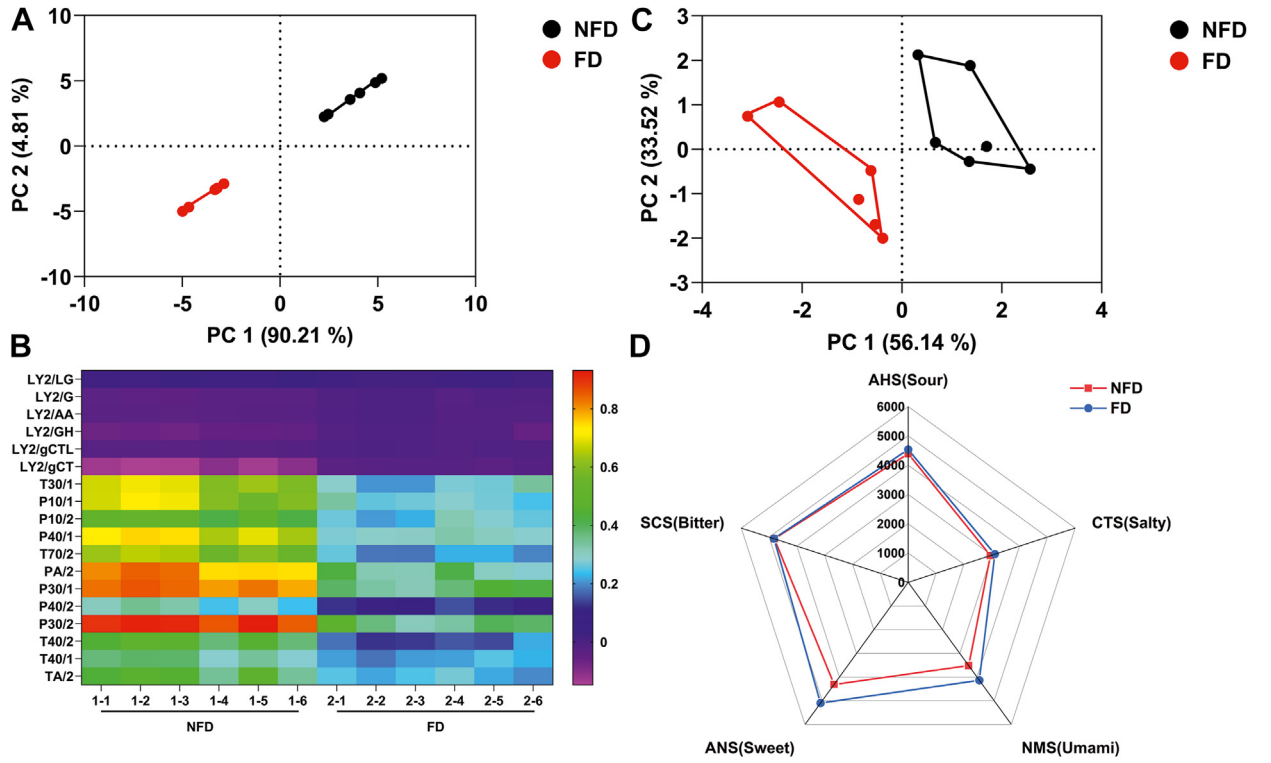

Fig. 3. Electronic sensory analysis of the odor/taste of meat from *Eriocheir sinensis*. (A) Principal component analysis (PCA) results of crab meat samples based on electronic nose data. (B) Heatmap results of crab meat samples based on electronic nose data. (C) PCA results of crab meat samples based on electronic tongue data. (D) Radar chart results of crab meat samples based on electronic tongue data. NFD = non-fermented feed; FD = fermented feed.

Table 7
The contents, taste attributes, taste thresholds, and taste activity values (TAV) of free amino acids in *Eriocheir sinensis* meat (mg/g, wet basis).

Index	Taste attribute (+, pleasant; -, unpleasant)		NFD	FD	*t*-value	*P*-value	Taste threshold, mg/mL	NFD TAV	FD TAV
Asp[1]	Umami	+	0.30 ± 0.02	0.39 ± 0.02	−6.537	0.001**	1.0	0.30	0.39
Glu[1]	Umami	+	0.76 ± 0.01	0.81 ± 0.08	−1.140	0.298	0.3	2.53	2.70
Ser	Sweet	+	0.27 ± 0.03	0.62 ± 0.06	−10.193	0.000***	1.5	0.18	0.41
Gly[1]	Sweet	+	4.62 ± 0.22	4.74 ± 0.44	−0.484	0.645	1.3	3.55	3.65
Ala[1]	Sweet	+	4.44 ± 0.04	4.89 ± 0.34	−2.623	0.076	0.6	7.40	8.15
Pro	Sweet	+	2.35 ± 0.08	2.88 ± 0.30	−3.413	0.014*	3.0	0.78	0.96
Thr	Sweet	+	1.07 ± 0.06	1.19 ± 0.07	−2.675	0.037*	2.6	0.41	0.46
Arg	Sweet	+	6.38 ± 0.25	6.72 ± 0.31	−1.747	0.131	0.5	12.76	13.44
Total pleasant free amino acids			20.19 ± 0.43	22.24 ± 0.57	−5.708	0.001**			
Val	Bitter	−	0.30 ± 0.02	0.50 ± 0.08	−4.909	0.003**	0.4	0.75	1.25
Tyr[1]	Bitter	−	0.28 ± 0.03	0.44 ± 0.06	−5.008	0.002**	ND	–	–
Cys	Bitter	−	0.20 ± 0.01	0.21 ± 0.03	−0.822	0.442	ND	–	–
Met	Bitter	−	0.22 ± 0.03	0.26 ± 0.02	−2.436	0.051	0.3	0.73	0.86
Ile	Bitter	−	0.21 ± 0.03	0.30 ± 0.02	−5.073	0.002**	0.9	0.23	0.33
Leu	Bitter	−	0.45 ± 0.04	0.47 ± 0.04	−0.891	0.407	1.9	0.24	0.25
Phe[1]	Bitter	−	0.41 ± 0.02	0.60 ± 0.05	−6.906	0.000***	0.9	0.46	0.67
Lys	Bitter	−	0.64 ± 0.01	0.65 ± 0.01	−1.459	0.195	0.5	1.28	1.30
His	Bitter	−	0.49 ± 0.03	0.50 ± 0.02	−0.316	0.762	0.2	2.45	2.50
Total unpleasant free amino acids			3.21 ± 0.10	3.96 ± 0.08	−11.604	0.000***			
Total free amino acids			23.40 ± 0.42	26.20 ± 0.63	−7.416	0.000***			

NFD = non-fermented feed; FD = fermented feed; ND = not detected.
Independent sample *t*-tests were used to analyze significance ($P < 0.05$).
The data were expressed as mean ± standard deviation ($n = 4$).
* Correlation was significant at $P < 0.05$.
** Correlation was significant at $P < 0.01$.
*** Correlation was significant at $P < 0.001$.
[1] "Delicious" amino acid.

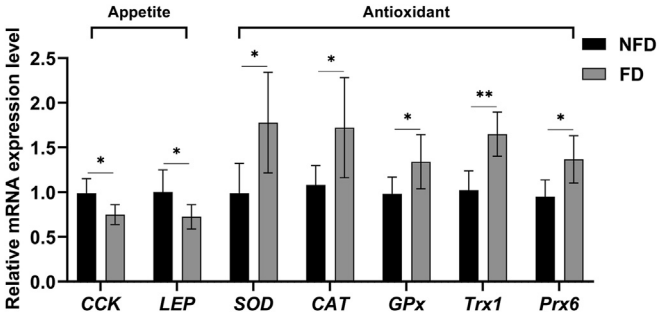

Fig. 4. The mRNA expression levels of appetite and antioxidant related genes of *Eriocheir sinensis*. Independent sample *t*-tests were used to analyze significance ($P < 0.05$). The data were expressed as mean ± standard deviation ($n = 4$). * Correlation was significant at $P < 0.05$. ** Correlation was significant at $P < 0.01$. NFD = non-fermented feed; FD = fermented feed.

Table 8
Alpha diversity analysis on intestinal microbiota of *Eriocheir sinensis* fed with different experimental feeds.

Index	NFD	FD	*Q*-value	*P*-value
Richness estimator				
Chao	167.11 ± 31.93	163.57 ± 38.21	0.865	0.865
Diversity estimators				
Shannon	1.32 ± 0.61	2.30 ± 0.20	0.022	0.004**
Simpson	0.52 ± 0.25	0.20 ± 0.03	0.031	0.011*

NFD = non-fermented feed; FD = fermented feed.
Independent sample *t*-tests were used to analyze significance ($P < 0.05$).
The data were expressed as mean ± standard deviation ($n = 4$).
* Correlation was significant at $P < 0.05$.
** Correlation was significant at $P < 0.01$.

Lactic acid produced during fermentation can cause a "sweet" and "sour" aroma in FD, improving the palatability of the feed and promoting the appetite of the feeding animals. Therefore, changes in lactic acid by fermentation were a key evaluation parameter (Chen et al., 2010; Wang et al., 2014). We speculated that the dramatic increase of lactic acid in FD (Table 4) was mainly due to the reproduction of *L. plantarum* subsp. *plantarum* in the second step of anaerobic fermentation. This phenomenon is identical to that reported in previous studies of *L. plantarum* fermentation (Yoon et al., 2005; Zhang and Vadlani, 2015). Of course, lactic acid is not the only attractant produced during fermentation: many others can be produced by fermented extracts, feeds, and broths (Li et al., 2020b). Previous research in aquaculture has mainly examined the attractiveness of feed based on the feed intake and the expression of appetite-regulating hormones. The feed choice comparison method applied in our study revealed significant differences in the behavioral aspect of crabs toward different feeds. Overall, crabs preferred FD over NFD (Fig. 2), demonstrating the attractant additive/saving potential of FD for crustaceans.

Quality evaluation of FD is not comprehensive enough to predict its effects on animal breeding, so culturing experiments of *E. sinensis* should also be carried out to evaluate growth performance, digestive enzyme activity, antioxidant capacity, meat flavor, and intestinal flora. In other species, animals fed with FD display better growth performance than those fed with conventional feeds, including piglets (Wang et al., 2014), laying hens (Zhu et al., 2020), turbot (Li et al., 2020a), and tilapia (Hassaan et al., 2018), etc. The current study also demonstrated that the FD promoted the growth of crabs more than the NFD (Table 5). In addition to the improvement of feed nutrition by fermentation, the decomposition of carbohydrates and the stronger feed attractiveness of FD might influence the growth performance of crabs as well. Furthermore,

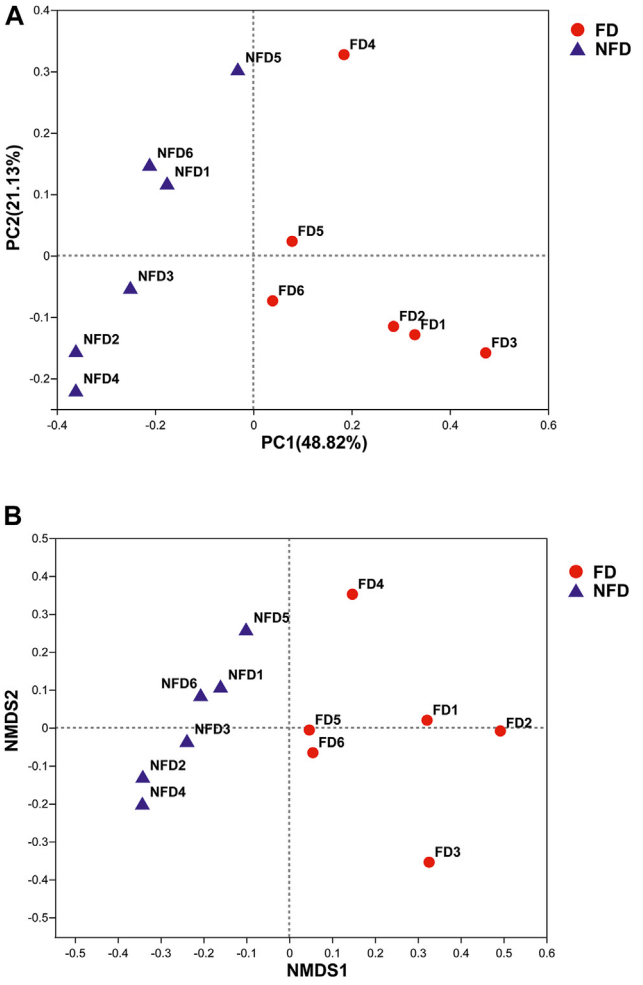

Fig. 5. Beta diversity analysis on intestinal microbiota of *Eriocheir sinensis*. (A) Unweighted principal coordinate analysis (PCoA) of UniFrac distances. (B) Unweighted nonmetric multidimensional scaling (NMDS) of UniFrac distances. NFD = non-fermented feed; FD = fermented feed.

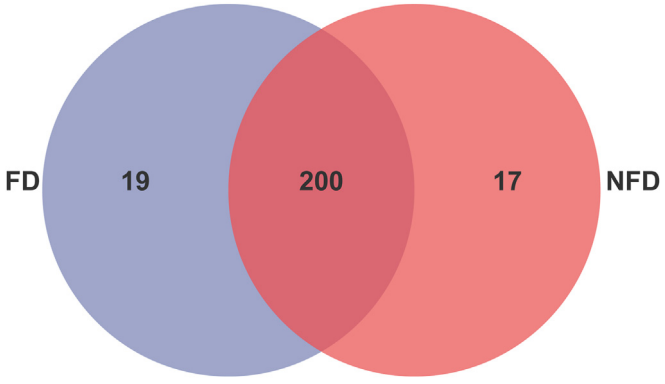

Fig. 6. Venn diagram of operational taxonomic units on intestinal microbiota of *Eriocheir sinensis*. NFD = non-fermented feed; FD = fermented feed.

many studies also reported that probiotics (supplemented directly) could improve growth performance and feed utilization of aquatic animals (Wang and Xu, 2006; Sahlmann et al., 2019; Xu et al., 2021). In line with our study, Pacific white shrimp (Zhang et al., 2021) and sea cucumber (Wang et al., 2017), which are also slow-foraging aquatic animals, showed better growth performance when fed an

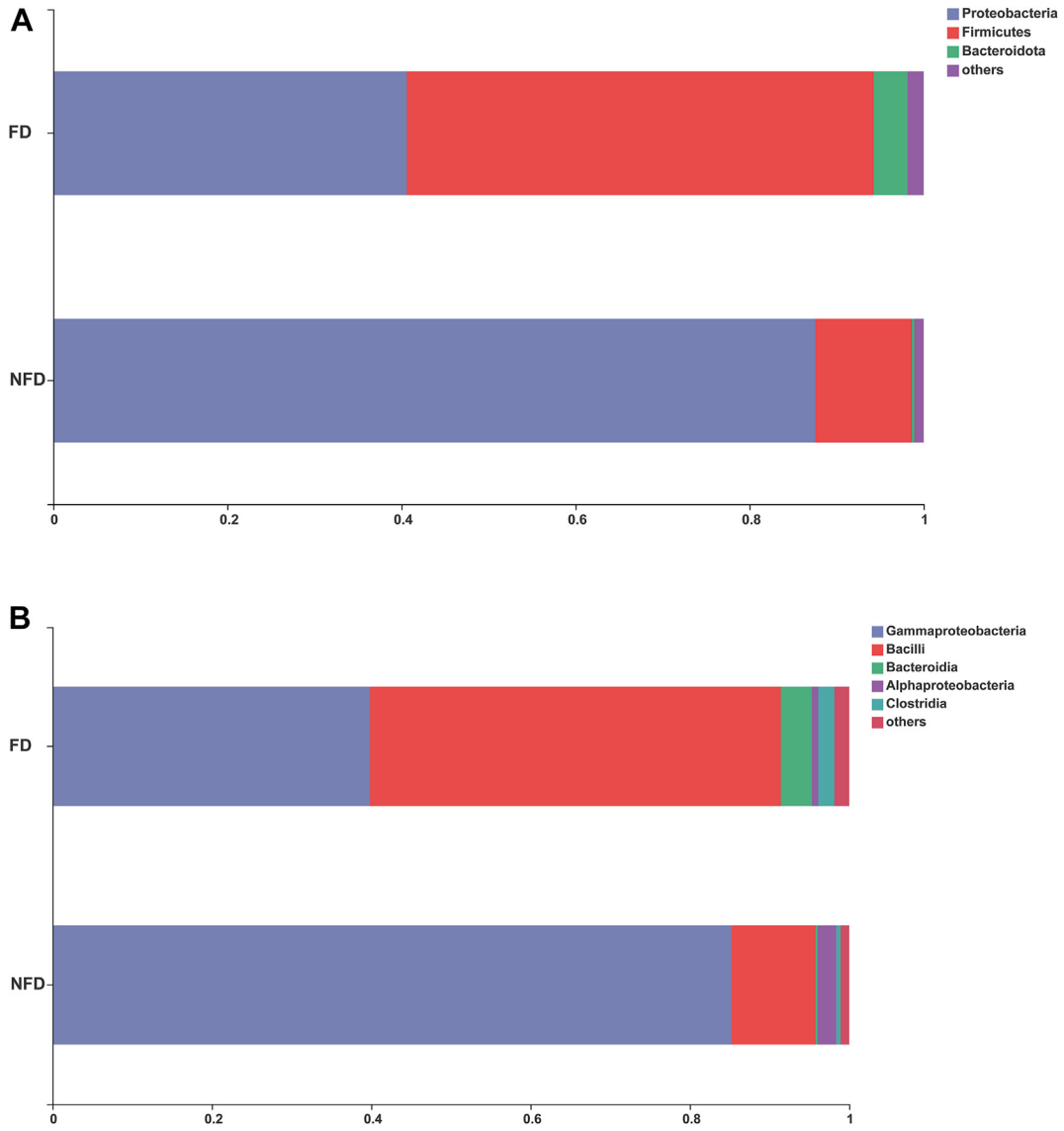

Fig. 7. The abundance of intestinal microbiota at phylum and class levels of *Eriocheir sinensis*. (A) The abundance of intestinal microbiota composition at phylum level. (B) The abundance of intestinal microbiota composition at class level. NFD = non-fermented feed; FD = fermented feed.

FD with higher crude protein and amino acid contents, and reduced carbohydrate.

The other aspect which possibly contributed to the enhanced growth performance of crabs was their improved digestive ability activated by digestive enzymes. Carrillo-Farnés et al. (2007) have suggested that the synthesis, secretion and regulation of digestive enzymes of crustaceans define their digestive capacities and depend essentially on the ingredients in the diet. The activity of protease has been shown to be crucial in the protein digestion process, and it was strongly modulated by the quality and quantity of dietary proteins (Sainz Hernández and Cordova Murueta, 2009). Lipase is a key enzyme in the absorption of long-chain triacylglycerol (Bitou et al., 1999). Omont et al. (2021) have also shown an increase in lipase activity in shrimp hepatopancreas fed with pre-digested *Ulva lactuca* that was associated with better lipid absorption. Amylase is a carbohydrase that plays an important role in crustaceans' digestion (Omont et al., 2021) as it can hydrolyse complex carbohydrates used as energy sources and growth promoters (Castro et al., 2012). According to Zhang et al. (2021), shrimp fed with FD generally exhibited higher digestive enzyme activities and better growth performance. Similarly, the crabs fed with FD showed stronger enzyme activities of protease, amylase, and lipase. We concluded that with the improvement of feed fermentation (such as higher contents of crude protein and amino acids, and degradation of carbohydrates and crude fiber), the activity of digestive enzymes in aquatic animals was enhanced, thereafter providing more energy for organisms. Generally, the growth performance of aquatic animals was positively related to digestive enzyme activities (Jannathulla et al., 2019; Omont et al., 2021; Zhang et al., 2021).

During evolution, animals developed a complex network of antioxidant defense systems to cope with the oxygenated atmosphere (Surai et al., 2019), including an endogenous enzyme-based antioxidant system, e.g., SOD, CAT, and GPx, and an exogenous, non-enzyme-based antioxidant system, e.g., vitamin C, vitamin E, glutathione, zinc, and selenium. T-AOC is a comprehensive index that can reflect the antioxidant capacity of both internal and external antioxidant defense systems (Bartosz, 2003). Antioxidant enzymatic scavengers in the internally synthesized antioxidant defense system, such as SOD, CAT, and GPx, play vital roles in

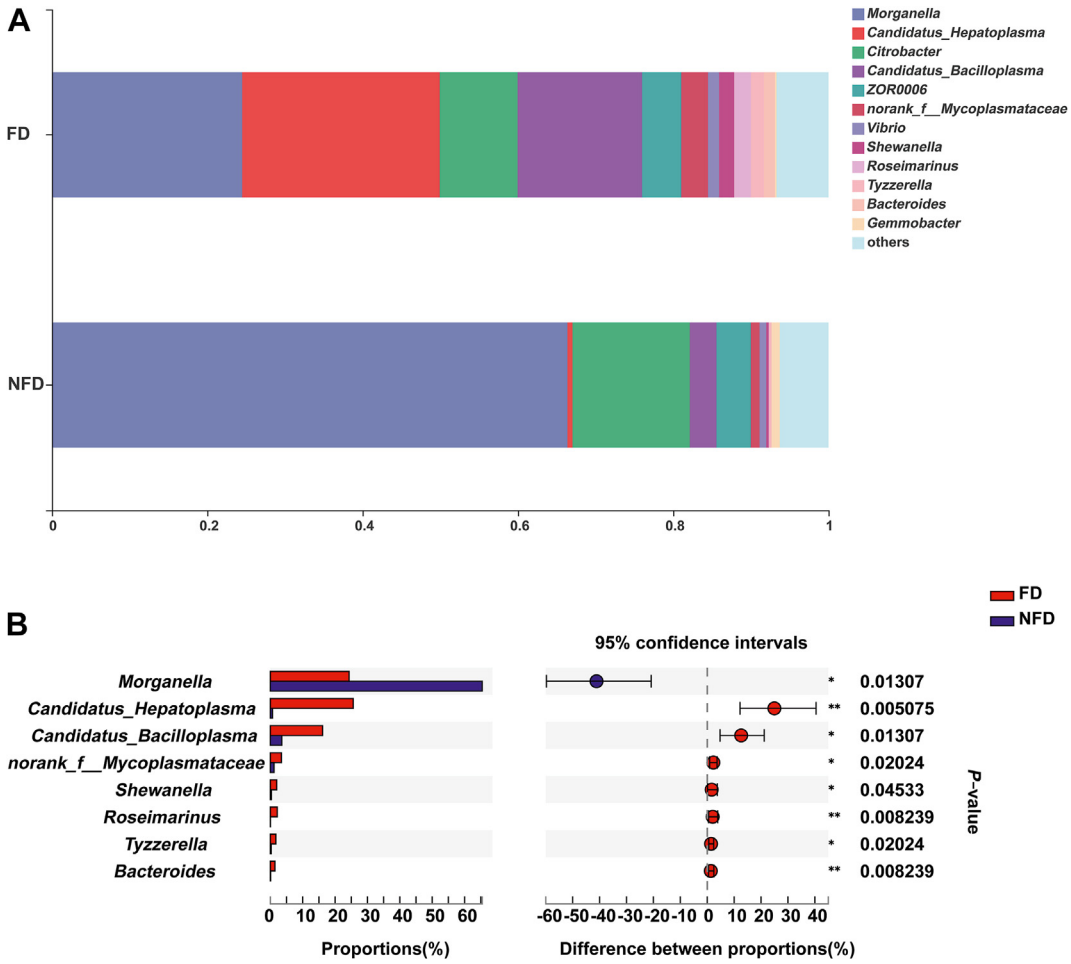

Fig. 8. The abundance of intestinal microbiota at genus level of *Eriocheir sinensis*. (A) The abundance of intestinal microbiota at genus level; (B) The difference between intestinal microbiota proportions at genus level. NFD = non-fermented feed; FD = fermented feed. Independent sample t-tests were used to analyze significance ($P < 0.05$). The data were expressed as mean ± standard deviation ($n = 4$). * Correlation was significant at $P < 0.05$. ** Correlation was significant at $P < 0.01$.

eliminating harmful reactive oxygen derivatives and oxygen toxicity produced by reactive oxygen species (ROS) in organisms (Ighodaro and Akinloye, 2018). Excessive ROS causes biological damage to organisms, mainly to lipids, proteins, and DNA, eventually leading to lipid peroxidation and protein carbonylation (Ighodaro and Akinloye, 2018). PCO forms rapidly during protein carbonylation, and can be detected in plasma with long-term stability. MDA is the lipid peroxidation product of polyunsaturated fatty acids in cell membrane structures (Pirinccioglu et al., 2010). Hence, the concentrations of PCO and MDA are markers of lipid peroxidation and protein carbonylation, respectively. Our results showed that FD-fed crabs had higher antioxidant capacity in the hemolymph, higher T-AOC levels, and higher enzyme activities of SOD, CAT, and GPx, as well as lower concentrations of PCO and MDA (Table 6). The antioxidant capacity results confirmed previous reports that FD facilitates antioxidation (Li et al., 2020a; Zhu et al., 2020).

In addition to the growth and health conditions, changes in flavor quality may directly determine whether FD-fed crabs are accepted by the market. The odor of crab meat is a sensory characteristic that emerges from a variety of volatile odorants, including aromatic hydrocarbons, alcohols, ketones, aldehydes, esters, nitrogen-containing, and other compounds (Gu et al., 2013; Cheng et al., 2019). Aromatic hydrocarbons have high olfactory thresholds and make little contribution to the overall odor (Maggiolino et al., 2019). Alcohols generally produce mild smells like aromas of fruits or plants and their olfactory threshold is also high (Pan and Kuo, 1994; Brewer, 2009). Notably, aldehydes and esters, commonly related to oxidation and metabolism of lipids, have relatively low olfactory thresholds and generally contribute sweet smells, which are important to the flavor of crab meat (Pan and Kuo, 1994; Gu et al., 2013; Cheng et al., 2019). Nitrogen-containing compounds, such as trimethylamine and trimethylamine oxide, are the main contributors to the formation of fishy odor (Amoore and Forrester, 1976). Su et al. (2020) documented that FD intake increased the contents of acid compounds, esters, ketones, and aldehydes in sheep muscle. In our study, crabs fed with NFD and FD had distinguishable meat odor (Fig. 3A); meat of crabs fed with FD had lower polar compounds, nonpolar compounds (hydrocarbons, ammonia, chlorine), aromatic (toluene, xylene), and amine odors (Fig. 3B). Although electronic-nose sensors can only identify large classes of substances, which are not specific enough, considering the classification of volatile odorants we concluded that the meat of FD-fed crabs had fewer fishy and irritating odors.

The taste substances in meat mainly produce 5 basic taste sensations including sweet, bitter, sour, salty, and umami (Farmer, 1999; Kurihara, 2015). The sour taste is contributed by organic acids, inorganic acids, and free amino acids. Moreover, the sweet taste is formed by saccharides and free amino acids (Sun et al., 2021). Meanwhile, the bitter taste comes from organic acid salts,

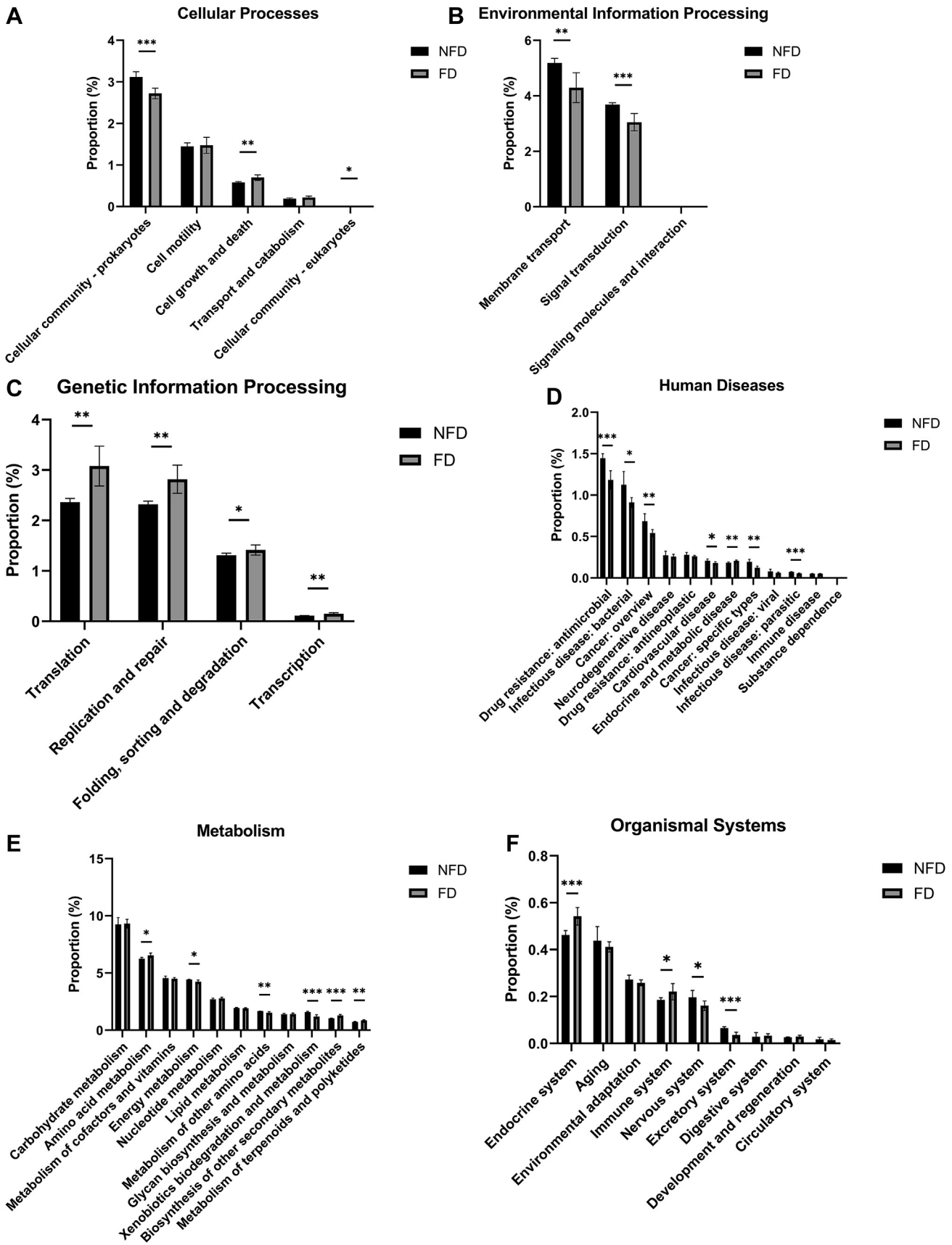

Fig. 9. Relative abundance of predicted functions matching in KEGG database at level 2. (A) Cellular processes; (B) Environmental information processing; (C) Genetic information processing; (D) Human diseases; (E) Metabolism; (F) Organismal systems. NFD = non-fermented feed; FD = fermented feed. Independent sample *t*-tests were used to analyze significance ($P < 0.05$). The data were expressed as mean ± standard deviation ($n = 4$). * Correlation was significant at $P < 0.05$. ** Correlation was significant at $P < 0.01$. *** Correlation was significant at $P < 0.001$.

inorganic acid salts, hypoxanthine, and free amino acids (Hayashi et al., 2013). Inorganic salts, sodium glutamate, terpenes, and bitter peptides can form a salty taste (Schulkin, 1982). The umami taste has been defined last in recent decades. It comes from 5′-nucleotides and their derivatives, glutamate, and sodium glutamate (Kurihara, 2015). It was clear that crabs fed with FD had a distinguishable meaty taste, manifested as improved sweet and umami sensations, compared with those provided with NFD in the current study (Fig. 3C and D). Tanaka et al. (2016) also reported similar improvements in these 2 tastes (by detecting concentrations of free amino acids) in carp muscle when fed with fermented products. As a critical non-volatile flavor-active substance in meat, the free amino acid profiles might help us understand the taste changes (Nishimura and Kato, 1988).

In general, the content and structure of free amino acids in the Chinese mitten crab meat in our study (Table 7) were similar to those reported by Chen and Zhang (2007); Chen et al. (2007). Arg, Gly, Ala, Pro, and Thr are the major free amino acids, accounting for more than 80% of total amino acids. The flavor of amino acids is closely related to the hydrophobicity of side chain R groups. When hydrophobicity is low, amino acids present with a sweet taste, such as Gly, Ala, Ser, and Pro, whereas when it is high, such as Leu, Ile, Lys, Arg, Tyr, and His, tastes are mainly bitter. Sour tastes appear when the side chain group is acidic, such as in Asp and Glu. The latter 2 amino acids are also important prerequisite substances for forming the “delicious” substances (Nishimura and Kato, 1988). Although 6 “delicious” amino acids have an umami taste, they may also contribute to unpleasant seafood flavors, such as Phe and Tyr; by contrast, Arg generally exhibits a pleasant, rather than a bitter taste (Zhao et al., 2019). Another point worth noting is that even if the content of some amino acids is low, their content can strongly contribute to taste, owing to low taste thresholds. It is generally accepted that amino acids contribute to meat taste when their TAVs >1 (Nishimura and Kato, 1988; Chen and Zhang, 2007). Our results (Table 7) show that the content and TAVs of free amino acids tended to be higher in FD-fed than in NFD-fed crabs, which indicated a richer flavor in the meat of FD-fed crabs. There were 4 pleasant-tasting amino acids (Glu, Gly, Ala, and Arg) and 3 unpleasant-tasting amino acids (Val, Lys, and His) in the FD group that showed a TAV of more than one. The TAV value of Ala and Arg is several times that of other taste-active substances, which contributes a lot to the taste. Val was the only significantly different amino acid with a TAV of more than 1, but its increment seemed inappreciable compared with the other 6 amino acids. As for Lys and His, it was reported that His could enhance the “meat flavor” of aquatic products (Yang et al., 2019). Meanwhile, Gly was also proved to inhibit bitter taste in some forms (Roland et al., 2011; Rhyu et al., 2020), so often, the bitter taste of the Chinese mitten crab was not obvious when consumed. Due to the limited sample size, we only conducted preliminary electronic sensory-based meat analysis and free amino acid detection. As things stand, the compositions of free amino acids in crabs were positively related to the indices of the electronic tongue.

To further verify the feed attractiveness and antioxidant capacity results obtained by using behavioral and biochemical methods, we measured their gene expression levels (Fig. 4). Some genes have important effects on the feeding and growth of aquatic animals: *LEP* and *CCK* have been extensively researched. CCK is a brain-gut peptide secreted in the gastrointestinal tract, while leptin is an obesity-associated adipokine that plays an important role in regulating appetite. They are considered satiety-inducing factors, affecting feed intake, feeding time, and eating termination in fish (Bertucci et al., 2019). Dockray (2012) pointed out that the expression of these 2 genes was negatively correlated with appetite. Jia et al. (2018) found that low *LEP* expression in the hepatopancreas and low *CCK* expression in the intestine increased the appetite of *E. sinensis*. Our results showed that the expression of *LEP* and *CCK* were significantly lower in the FD group than the NFD group, indicating the higher appetites of crabs fed with FD. Regarding to antioxidant genes, mRNA expression levels of *SOD*, *CAT*, and *GPx* in hepatopancreas tissue were upregulated in the FD group, which was consistent with their higher enzyme activities in the hemolymph (Table 6). A similar correlation between antioxidant enzyme activity and gene expression was reported recently in a study of the effect of *Aeromonas hydrophila* infection in *E. sinensis* (Zheng et al., 2022). Beyond that, the finding that FD strengthened the antioxidant capacity in crabs could also be further verified by the increased expression levels of *Trx1* and *Prx6* of the FD-fed crabs than those fed with NFD. *Trx1* and *Prx6* were previously found to be efficient in the antioxidant response and relieve oxidative stress in *E. sinensis* (Zheng et al., 2019; Wen et al., 2021). Song and Roe (2008) pointed out that *Trx1* regulates cellular redox homeostasis under physiological conditions by activating the thioredoxin system and has a higher antioxidant capacity than GSH. As for *Prx6*, it belongs to the superfamily of non-selenium peroxidases. Several lines of evidence suggest that it plays a physiologically important role in the enzymatic removal of ROS and in protecting the organism cells against highly reactive oxidative stress (Mu et al., 2009). The increased expression levels of *Trx1* and *Prx6* in our study suggested a better ability of these crabs to maintain the redox balance of their organism.

The intestinal microbiota has drawn much attention recently due to its crucial role in host health. The composition and abundance of the intestinal microbiota can be affected by feed ingredients and are important factors that affect hosts (Li et al., 2020a; Zhang et al., 2021). In the present study, FD increased the microbiota diversity, although it had no significant influence on the richness (Table 8). Consistent with this study, Han et al. (2022) also found that partial replacement of soybean meal with fermented cottonseed meal in a low fishmeal diet enhanced microbiota diversity but not richness in the shrimp gut. It may be because the types of microorganisms in the experimental environments were relatively similar and stable. Fermented feed might help to enhance the colonization of probiotics, improve microecological balance, and benefit the host intestinal environment (Dawood and Koshio, 2019). Beta diversity analysis was performed to analyze the similarity of the bacterial community structures (Fig. 5). In previous studies, clear shifts in microbial community structure observed by PCoA and NMDS indicated great changes in intestinal microbiota under different conditions (Hou et al., 2018; Li et al., 2022a). The significant differentiation of samples in PCoA and NMDS in this study demonstrated the great influence of dietary FD on the intestinal community structure of crabs.

Our data showed that the dominant OTUs in crabs were distributed in Proteobacteria and Firmicutes (Fig. 7A). Numerous reports have shown that Proteobacteria is a dominant member of marine animals (Sun et al., 2018; Zhang et al., 2021), involved in the nutrient cycle and the mineralization of organic compounds (Liu et al., 2021). Sun et al. (2018) speculated that the decreased abundance of Proteobacteria may be correlated with the improved growth performance of juvenile swimming crabs, which was in agreement with the results of our research (Table 5). However, its function in crabs has not yet been extensively explored. Firmicutes was also reported to be the dominant phyla in the intestines of crustaceans, such as *E. sinensis* (Feng et al., 2021), *Scylla paramamosain* (Li et al., 2012), and *Macrobrachium rosenbergii* (Liu et al., 2021). Firmicutes contain lots of probiotics, such as *Lactobacillus*, *Enterococcus*, and *Bacillus*, which may help to defend against pathogens, improve digestion and immunity, and maintain the intestinal health of aquatic animals (Feng et al., 2021). With FD, the

Firmicutes in crab intestines increased dramatically in our study, which was consistent with the selection of fermentation strains and the health status of crabs (Table 6, Fig. 4). Meanwhile, we also assumed that the fermented feed could increase the possibility of probiotic colonization in the intestine of *E. sinensis*.

Moreover, at the genus level, there were several significant genera differences between the NFD and FD groups, while we mainly focused on the common beneficial and harmful intestinal bacteria genera. The abundance of *Morganella* was decreased significantly after feeding FD, whereas the abundances of *Candidatus_Hepatoplasma* and *Candidatus_Bacilloplasma* were increased significantly (Fig. 8). *Morganella* was known as a non-negligent opportunistic pathogen, and was reported to cause diseases in aquatic animals (Li et al., 2022b). Several studies have demonstrated that *Candidatus_Hepatoplasma* is a dominant genus in the intestines of *E. sinensis* (Feng et al., 2021; Li et al., 2022b). *Candidatus_Hepatoplasma* was documented to help facilitate nutrition uptake in *Takifugu obscurus* (Yang et al., 2007) and improve resistance to QX disease in *Saccostrea glomerata* (Nguyen et al., 2021). *Candidatus_Bacilloplasma* was recently reported to be implicated in shrimp diseases. It was reduced during white spot syndrome virus infection (Wang et al., 2019a) and was more abundant in healthy shrimp than in white feces syndrome ones (Wang et al., 2020). Together, a plausible explanation is that FD might cause alterations at the phylum and genus levels of the intestinal microflora; that is, FD might help to increase the content of beneficial flora and decrease the content of harmful bacteria. Thus, we extrapolated that FD improve the contribution of intestinal microbiota in the metabolism and disease resistance of *E. sinensis*.

Based on the functional PICRUSt prediction, the intestinal microorganisms of crabs were rich in metabolism functions, especially the amino acid and carbohydrate metabolic pathways (Fig. 9E), consistent with the findings in the metagenomic studies of intestines of crustaceans (Shao et al., 2019; Zhang et al., 2021). Gibson and Roberfroid (1995) have provided a possible reason that the host-derived proteins and carbohydrates are the primary nutrients for bacterial survival and colonization. Besides, diverse metabolism-related functional features of intestinal microbiota were detected between the 2 groups (Fig. 9 E). We found that the relative abundance of amino acid metabolism, biosynthesis of other secondary metabolites, and metabolism of terpenoids and polyketide pathways increased in the FD group compared with the NFD group. This phenomenon may be attributable to the large number of small molecule proteins and flavonoids in FD, to enable extra capability for biosynthesizing more metabolites of FD-fed crabs. On the contrary, the relative abundance of energy metabolism, and xenobiotics biodegradation and metabolism pathways were decreased. It might imply that, owing to the easier utilization of FD compared with NFD, the potential absorbability of some substances by the crabs was inhibited instead. As for other predicted functions, no homogeneous regular pattern was found in the changes of unigenes in our study, as previous studies on crustaceans have suggested (Feng et al., 2021; Zhang et al., 2021; Han et al., 2022). Overall, the intestinal microbiota regulates complex interspecies interactions of various host metabolic pathways through the microbial ecological network (Banerjee et al., 2018). In addition, the information regarding the function of intestinal microbiota in *E. sinensis* is currently limited, and further investigation is worthy of further study.

5. Conclusion

Compound feed fermentation could be a practical approach to enhance feed quality and benefit the rearing of *E. sinensis*. The nutritional quality of feed and the removal of ANFs were dramatically improved by fermentation. In addition, FD was more attractive and appetizing for crabs than NFD. Moreover, FD positively impacted the growth performance, antioxidant capacity, digestive enzyme activity, meat flavor, and intestinal microflora of *E. sinensis*.

Author contributions

Weibo Jiang: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft, Visualization. **Xiaoyan Jia**: Methodology, Software, Visualization. **Ningjun Xie**: Formal analysis, Methodology, Software. **Chuang Wen**: Formal analysis, Software, Validation. **Shuo Ma**: Formal analysis, Software, Validation. **Guangzhen Jiang**: Writing - review & editing. **Xiangfei Li**: Writing - review & editing. **Cheng Chi**: Writing - review & editing. **Dingdong Zhang**: Conceptualization, Supervision, Funding acquisition, Project administration, Resources, Writing - review & editing. **Wenbin Liu**: Supervision, Funding acquisition, Project administration, Resources.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that could 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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