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Effects of glycinin and β-conglycinin on growth performance and intestinal health in juvenile Chinese mitten crabs (*Eriocheir sinensis*)

Fenglu Han[a], Xiaodan Wang[a], Jianlin Guo[c], Changle Qi[a], Chang Xu[b], Yuan Luo[a], Erchao Li[b], Jian G. Qin[d], Liqiao Chen[a,*]

[a] Laboratory of Aquaculture Nutrition and Environmental Health, School of Life Sciences, East China Normal University, 500 Dongchuan Rd, Shanghai, 200241, China
[b] Department of Aquaculture College of Marine Sciences, Hainan University, Haikou, Hainan, 570228, China
[c] Agriculture Ministry Key Laboratory of Healthy Freshwater Aquaculture, Key Laboratory of Freshwater Aquaculture Genetic and Breeding of Zhejiang Province, Zhejiang Institute of Freshwater Fisheries, Huzhou, 313001, China
[d] College of Science and Engineering, Flinders University, Adelaide, SA, 5001, Australia

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ABSTRACT

This study investigates the effects of two soybean antigens (glycinin and β-conglycinin) as an antinutritional substance in the diet on the growth, digestive ability, intestinal health and microbiota of juvenile Chinese mitten crabs (*Eriocheir sinensis*). The isonitrogenous and isolipidic diets contained two soybean antigens at two levels each (70 and 140 g/kg β-conglycinin, 80 and 160 g/kg glycinin) and a control diet without β-conglycinin or glycinin supplementation, and were used respectively to feed juvenile *E. sinensis* for seven weeks. Dietary inclusion of either glycinin or β-conglycinin significantly reduced crab survival and weight gain. The crabs fed diets containing soybean antigens had higher malondialdehyde concentrations and lower catalase activities in the intestine than those in the control. The activities of trypsin and amylase in the intestine were suppressed by dietary β-conglycinin and glycinin. Dietary glycinin or β-conglycinin impaired the immunity and morphological structure of intestine, especially the peritrophic membrane. The mRNA expression of constitutive and inducible immune responsive genes (lipopolysaccharide-induced TNF-α factor and interleukin-2 enhancer-binding factor 2) increased while the mRNA expression of the main genes related to the structural integrity peritrophic membrane (peritrophin-like gene and peritrophic 2) significantly decreased in the groups with soybean antigen addition. Soybean antigen could also change the intestinal microbial community. The abundance of pathogenic bacteria (*Ochrobactrum*, *Burkholderia* and *Pseudomonas*) increased significantly in both soybean antigen groups. Although pathogenic bacteria *Vibrio* were up-regulated in the glycinin group, the abundance of *Dysgonomonas* that degraded lignocellulose and ameliorated the gut environment decreased in the glycinin group. This study indicates that existence of soybean antigens (glycinin or β-conglycinin) could induce gut inflammation, reshape the community of gut microbiota, and cause digestive dysfunction, ultimately leading to impaired growth in crabs.

1. Introduction

Fishmeal replacement by plant protein in the diet has been an active research field in aquatic animal nutrition in the past two decades, and soybean meal is one of the mostly used ingredients to replace fishmeal because of its well-balanced amino acid composition [1]. Soybean meal contains essential amino acids, vitamins, and other nutrients required by aquatic animals, but it cannot completely replace fishmeal in most aquafeed because soybean meal contains anti-nutritional factors such as allergens, trypsin inhibitor and saponins [2]. Excessive soybean meal in a diet can decrease hepatopancreatic trypsin activity in *Portunus pelagicus* [1], reduce the activity of antioxidant enzymes in *Litopenaeus vannamei* [3], and inhibit animal growth [1,3]. These negative effects are partly due to the existence of anti-nutritional factors such as glycinin and β-conglycinin [4].

Digestive and absorptive functions are closely related with the structural integrity and digestive enzymes in the intestine [5]. However, both glycinin and β-conglycinin can interfere the digestive enzyme activities in fish [5–7]. Either glycinin or β-conglycinin in the diet can decrease intestinal digestive enzymes in Jian carp (*Cyprinus carpio* var. Jian) [5,6], and β-conglycinin can lower villi heights and cause fusion of mucosal fold in the intestine of turbot (*Scophthalmus maximus*

* Corresponding author.
E-mail address: lqchen@bio.ecnu.edu.cn (L. Chen).

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L.) [7,8]. Unlike fish, the peritrophic membrane is a special structure in the gut of crustacean to coat the surface of intestinal epithelial cells for protection of chemical or mechanical damage [9], though our understanding on the role of the peritrophic membrane is currently limited to the level of morphological structure.

Other than damaging intestinal structure, anti-nutritional factors in the diet could also cause intestinal inflammation [5,6] and lead to production of excessive reactive oxygen species (ROS) [10]. The soy antigen is a type of anti-nutritional factor and can cause intestinal damage and inflammatory disorder in piglets [11]. In Jian carp, glycinin and β-conglycinin increased the mRNA expression of pro-inflammatory, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), and decreased the mRNA expression of anti-inflammatory, transforming growth factor-β (TGF-β) in the distal intestine [5,6]. Furthermore, lipopolysaccharide-induced tumor necrosis factor-α (LITAF) is a transcription factor participated in intestinal inflammation [12] and its gene level in the gut of *Litopenaeus vannamei* can be significantly increased by dietary soybean meal [13].

Some digestive functions are achieved through the process of bacterial metabolism in the digestive tract [14,15]. The microbial composition in the intestine of aquatic animals can affect the health and growth of hosts through regulation of nutrient uptake, immune response and metabolism [16]. Due to the rapid response of bacteria to the changes in food intake and composition, diets containing different source of protein can affect the community structure of intestinal microbiota [17]. In northern snakehead (*Channa argus* Cantor, 1842), for instance, the abundance of intestinal probiotics in fish fed 700 g/kg soybean meal was significantly lower than those fed fishmeal [18]. As fermentation can reduce the activity of anti-nutritional factors in soybean meal [19], the intestine of Atlantic salmon (*Salmo salar*) fed fermented soybean meal showed higher lactic acid production and had a better ability of digestion and absorption than those fed either a soybean meal diet or a fishmeal diet [17].

The Chinese mitten crab (*Eriocheir sinensis*) is an important economic species in China, and the production was over 800 000 tons in 2015 [20]. Due to limited supply and high price of fishmeal, more and more plant protein has been used to replace fishmeal in aquaculture feed. However, with the increasing inclusion of soybean meal in the diet, *E. sinensis* has faced a health challenge. The aim of this study was to investigate the negative effects of glycinin and β-conglycinin on growth performance, digestive ability, intestinal health and microbiota of juvenile Chinese mitten crabs. The results of the current study would provide insights into the understanding on the dose effect of glycinin and β-conglycinin on the intestinal health in crustacean species and offer insight information for the replacement of fishmeal with soybean meal in *E. sinensis* diets.

2. Materials and methods

2.1. Experimental diets

Purified soybean β-conglycinin and glycinin used in this study were provided by the Food Institute of Jiangnan University. Purity coefficient of glycinin and β-conglycinin was 92.3% and 70.2%, respectively. Five isonitrogeous and isolipidic experimental diets were formulated, and the ingredients and nutrient composition of theexperimental diets are shown in Table 1. The proteins in the control diet (C) included fish meal, casein and gelatin. The other four diets were supplemented with 70 or 140 g/kg β-conglycinin, and 80 or 160 g/kg glycinin. The dose of 70 g/kg β-conglycinin and 80 g/kg glycinin was equivalent to the protein amount of 400 g/kg de-hulled soybean meal. While the dose of 140 g/kg β-conglycinin and 160 g/kg glycinin was equivalent to the protein amount of 800 g/kg de-hulled soybean meal. The diets were named as C, 7S (70 g/kg β-conglycinin), 7S+ (140 g/kg β-conglycinin), 11S (80 g/kg glycinin), 11S+ (160 g/kg glycinin). The feed pellets were extruded into 1.5-mm diameter by a double helix plodder (F-26, South China University of Technology Industrial Factory, Guangdong, China). During the extruding process when the diets were made, the temperature of the extruder die was 50–60 °C, which may help improve the starch digestibility in some way. And then dried through blowing air at room temperature to contain < 10% moisture. The diets were stored at −20 °C until feeding.

Table 1
Ingredient formulation (g Kg^{-1} dry basis) and proximate composition (%) of the five experimental diets fed to *Eriocheir sinensis*.

	C	7S	7S+	11S	11S+
Fish meal	200	200	200	200	200
Casein	205	135	60	120	35
Gelatine	50	50	50	50	50
7S(70%)	0	70	140	0	0
11S(92%)	0	0	0	80	160
Corn starch	270	270	270	270	270
Arginine[a]	18.8	15.8	13	15.6	12.3
Lysine[a]	0	0	0	0	1.2
Methionine[a]	1.4	0	0	2.7	4
Threonine[a]	1.2	2	3.4	2.1	0
Fish oil	20	20	20	20	20
Soybean oil	25	25	25	25	25
lecithin	5	5	5	5	5
Vitamin premix[b]	30	30	30	30	30
Mineral premix[c]	20	20	20	20	20
CMC	20	20	20	20	20
Choline chloride	5	5	5	5	5
Betaine	10	10	10	10	10
Cellulose	113.1	116.7	123.1	119.1	127
Total	1000	1000	1000	1000	1000
Analyzed proximate composition					
Moisture	10.02	10.69	10.11	10.53	10.36
Crude protein	37.90	38.84	38.15	38.18	37.94
Crude lipid	8.25	8.04	8.14	8.93	8.51
Ash	7.48	7.59	8.73	7.75	8.20

[a] Sangon Biotech, Ltd., Shanghai, China.

[b] Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; pyridoxine hydrochloride, 0.225 g; para-aminobenzoic acid, 0.1 g; ascorbic acid, 0.5 g; biotin, 0.005 g; folic acid, 0.025 g; cholecalciferol, 0.0075 g; α-tocopherol acetate, 0.5 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g.

[c] Mineral premix (per 100 g premix): KH_2PO_4, 21.5 g; NaH_2PO_4, 10.0 g; $Ca(H_2PO_4)_2$, 26.5 g; $CaCO_3$, 10.5 g; KCl, 2.8 g; $MgSO_4{\cdot}7H_2O$, 10.0 g; $AlCl_3{\cdot}6H_2O$, 0.024 g; $ZnSO_4{\cdot}7H_2O$, 0.476 g; $MnSO_4{\cdot}6H_2O$, 0.143 g; KI, 0.023 g; $CuCl_2{\cdot}2H_2O$, 0.015 g; $CoCl_2{\cdot}6H_2O$, 0.14 g Calcium lactate, 16.50 g; Fe-citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g.

2.2. Experimental crab and rearing

Juvenile *E. sinensis* were purchased from Shanghai Ocean University, China, and acclimated in 300-L tanks for one week at the Experimental Base of Zhejiang Freshwater Fisheries Research Institute (Huzhou, China). Thirty-five crabs (1.17 ± 0.03 g) with intact appendages were randomly assigned to each tank with five replicates (25 tanks in total). Four groups of corrugated plastic pipes (12 cm long and 25 mm diameter with six pipes in each group) and four arched tiles were placed in each tank as shelters to reduce attacking behavior. The daily ration of 4% body mass was split into three meals fed at 07:00 (20%), 16:00 (20%) and 21:00 h (60%) over seven weeks. Feces and uneaten feed were removed 2 h after feeding, and the water of 50% tank volume was exchanged daily. The incoming freshwater in the experiment was filtered through a quartz sand filter (Xinyi Water Treatment Equipment Factory, Huzhou, China) and aerated fully before entering the culture system. During the whole trial, dead crabs were removed and recorded for each individual weight. Water pH ranged from 7.6 to 8.4 and temperature fluctuated from 25.0 to 28.0 °C. Dissolved oxygen was maintained at > 7.0 mg/L, whereas ammonia-N

was kept at < 0.05 mg/L.

2.3. Sample collection

At the end of the 7-week feeding experiment, crabs were fasted for 24 h. All crabs were counted and individually weighed. Subsequently, all crabs were anesthetized on ice. Three crabs per tank were stored at −20 °C for the analysis of whole body composition. Hemolymph was sampled with a 1-mL syringe on the leg joints of nine crabs in each tank. After incubation at 4 °C for 24 h, serum was separated from the hemolymph by centrifugation (5415 R, Eppendorf, Germany) at 4500 rpm and 4 °C for 10 min and stored at −80 °C for enzyme activity analysis. The whole guts and hepatopancreas were rapidly removed from three crabs and stored at −80 °C until analysis. The hind-guts of the other three crabs from each tank were cut into sections with a same length and fixed in the buffered formalin before histological analysis. The mid-intestine from other three crabs was taken for microbiota analysis. The research protocol was approved by the Committee of the Ethics of Animal Experiments of East China Normal University.

2.4. Calculations and statistical analysis

Survival rate (SR), weight gain (WG) and specific growth rate (SGR) were calculated using the following formulae:

Survival rate (SR, %) = 100 × (final crab number/initial crab number)

Specific growth rate (SGR, % d^{-1}) = 100 × [Ln (final weight) − Ln (initial weight)] / days

Weight gain (WG, %) = 100 × [final weight – initial weight]/initial weight

2.5. Chemical composition analysis

The body composition of crabs and the proximate composition of diets were determined according to the standard methods (AOAC) [21]. Moisture was analyzed by drying the samples at 105 °C until a constant weight. Crude protein was measured by the Kjeldahl method using Kjeltec™ 8200 (Kjeltec, Foss, Sweden). Lipid was quantified by the method of Bligh and Dyer [22] using a vacuum drying oven (DZF-6050, Jinghong, Ltd, Shanghai, China). For ash content analysis, samples were placed in a muffle furnace (PCD-E3000 Serials, Peaks, Japan) at 550 °C for 8 h.

2.6. Digestive enzymes activities and intestine antioxidant capacity

Hepatopancreases and intestine were weighed and homogenized in 10 vol (v/w) of pre-chilled 0.85% saline solution and centrifuged at 1500 × *g* (5415R, Eppendorf, Germany) for 30 min at 4 °C. The resultant supernatant was collected and aliquots were stored at −80 °C until analysis. Amylase, trypsin, lipase and total protein in the hepatopancreases and intestine were measured with commercial assay kits (Cat. No. C016-1, A080-2, A054-1 and A054-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Glutathione (GSH), malondialdehyde (MDA), total superoxide dismutase (T-SOD), catalase (CAT) and glutathione S-transferase (GST) in the intestine were determined using commercial kits (Cat. No. A006-2, A003-1, A001-1-1, A007-2 and A004, Nanjing Jiancheng Bioengineering Institute) in accordance with the instructions of the manufacturer. As a result, enzyme activity units were expressed as the enzyme activity per mg of tissue protein.

Table 2
Primer pair sequences and product size of the genes used for real-time PCR (qPCR).

Gene	Position	5′-3′ Primer sequence	Length	Access No.
EsPT	Forward	CTTCCAACCCACGTCCAGTCT	21	KM433863
	Reverse	AACCAGCATCGGGACACCTTA	21	
EsPM1	Forward	CGCCGAGAAGTGTGACTACA	20	KU041138.1
	Reverse	GAAGTAGCCGTTCTGTCGCA	20	
EsPM2	Forward	CTCGTCGATGACCAAGGACC	20	KU041139.1
	Reverse	CCTCTGCGGGAAGGAAACTT	20	
EsLITAF	Forward	TCCATTCACACCTATTCAA	19	KF892539
	Reverse	TGGCAATGAGGACATATC	18	
EsRelish	Forward	TCAGGATTCGGTGGCAACTC	20	GQ871279
	Reverse	ATCTGCACTTGGACCGATGG	20	
p38MAPK	Forward	ATGTCGGAGGAACAACCCAC	20	KF582665.1
	Reverse	ATAGGCGCCTTCACCAATGT	20	
ILF2	Forward	GGGAACTTCGATGCCTGTCA	20	GU002546
	Reverse	ATGACCACGATGTCCGCTAC	20	
ALF3	Forward	TCTATGGCACAACGACACCG	20	HQ850572.1
	Reverse	TGCCCTCGTGGTACAATTCC	20	
β-actin	Forward	GCATCCACGAGACCACTTACA	21	KM244725.1
	Reverse	CTCCTGCTTGCTGATCCACATC	21	

2.7. Analysis of gene expression in mid-intestine

Total RNA was isolated from the mid-intestine of five replicates with the Trizol (RN0101, Aidlab, China). The purity of RNA was detected on a Nano Drop 2000 spectrophotometer (Thermo, USA). Only the RNA samples with the A260/A280 ratio between 1.8 and 2.0 were used for the subsequent analysis. A PrimeScript™ RT master mix reagent kit with a gDNA eraser (Perfect Real Time, Takara, Japan) was used to synthesize cDNA for quantitative real time-PCR (RT-qPCR).

The RT-PCR was performed with the CFX96 Real-Time PCR system (Bio-rad, Richmond, CA). The specific primers for the genes were designed with Primer Premier Software 6.0 according to the *E. sinensis* sequences (Table 2). The reactions were done in a volume of 10 μL containing 0.5 μL of 10 mM each of forward and reverse primers (1 mmol/L), 2.5 μL of diluted cDNA (1:5 diluted) and 5 μL 2 × SYBR Premix Ex TaqTM. The programmed reaction steps included 94 °C for 3 min, followed by 40 cycles at 94 °C for 15 s and 58 °C for 50 s, and 72 °C for 20 s. Data were quantified by the $2^{-\Delta\Delta CT}$ method [23] and were subjected to statistical analysis.

2.8. Histological assay

The intestine segments were dehydrated in ethanol, cleaned in toluene, equilibrated in xylene, and embed in paraffin to make solid wax blocks. Then, the embedded intestine was sectioned with a rotary microtome at approximately 5-μm thick and stained with hematoxylin and eosin. The tissue slices were stained with haematoxylin and eosin (HE) and observed on an Axioskop microscope (BX51, Olympus, Tokyo, Japan).

2.9. Analysis of the intestine microbiota

Total bacterial DNA was extracted from samples (C, 7S and 11S) using the DNA isolation kit (E.Z.N.A.™Soil DNA kit) according to the manufacturer's protocol. DNA quality and quantity were measured by the ratios of 260 nm/280 nm and 260 nm/230 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAG GCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWT-CTAAT-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR reaction system was conducted according to the reported protocol for *L. vanname* [24]. PCR

Table 3
Data from Illumina high-throughput sequencing yields bacterial diversity and richness based on operational taxonomic units (OTU), diversity index (Shannon & Simpson) and estimated OTU richness (Chao1 & ACE) for the intestinal bacterial diversity analysis of *Eriocheir sinensis* fed different experimental diets. ANOVA followed by Tukey's multiple comparison test was performed, where dissimilar letters represent significant difference ($P < 0.05$).

Diets	Sampling depth	Richness estimate		Diversity estimators	
	Mean sequence (bp)	Chao1	Ace	Shannon	Simpson
C	36178	863.25 ± 65.56	869.15 ± 71.87	6.54 ± 0.35	0.97 ± 0.01
7S	33746	881.08 ± 110.01	884.74 ± 111.26	6.66 ± 0.20	0.97 ± 0.01
11S	40153	990.31 ± 56.91	998.82 ± 57.66	6.65 ± 0.25	0.97 ± 0.01

Table 4
Growth performance and survival of *Eriocheir sinensis* fed the different experimental diets (mean ± SE).

	7S treatments			11S treatments		
	C	7S	7S+	C	11S	11S+
SR (%)	84.57 ± 0.70^{b}	75.43 ± 1.94^{a}	73.14 ± 1.47^{a}	84.57 ± 0.70^{b}	76.00 ± 2.49^{a}	73.14 ± 1.94^{a}
IW (g)	1.18 ± 0.01	1.17 ± 0.01	1.18 ± 0.01	1.18 ± 0.01	1.16 ± 0.01	1.17 ± 0.01
FW (g)	3.39 ± 0.03^{c}	2.81 ± 0.04^{b}	2.58 ± 0.03^{a}	3.39 ± 0.03^{b}	2.78 ± 0.04^{a}	2.70 ± 0.03^{a}
WG (%)	186.86 ± 2.54^{c}	139.96 ± 3.71^{b}	119.65 ± 3.40^{a}	186.86 ± 2.54^{b}	139.96 ± 5.51^{a}	130.89 ± 3.05^{a}
SGR (%d^{-1})	2.15 ± 0.02^{c}	1.78 ± 0.03^{b}	1.60 ± 0.03^{a}	2.15 ± 0.02^{b}	1.78 ± 0.05^{a}	1.71 ± 0.03^{a}

SR, survival rate; IW, average initial weight; FW, average final weight; WG, weight gain; SGR, specific growth rate.
The β-conglycinin treatments (7S, 7S+) and glycinin treatments (11S, 11S+) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

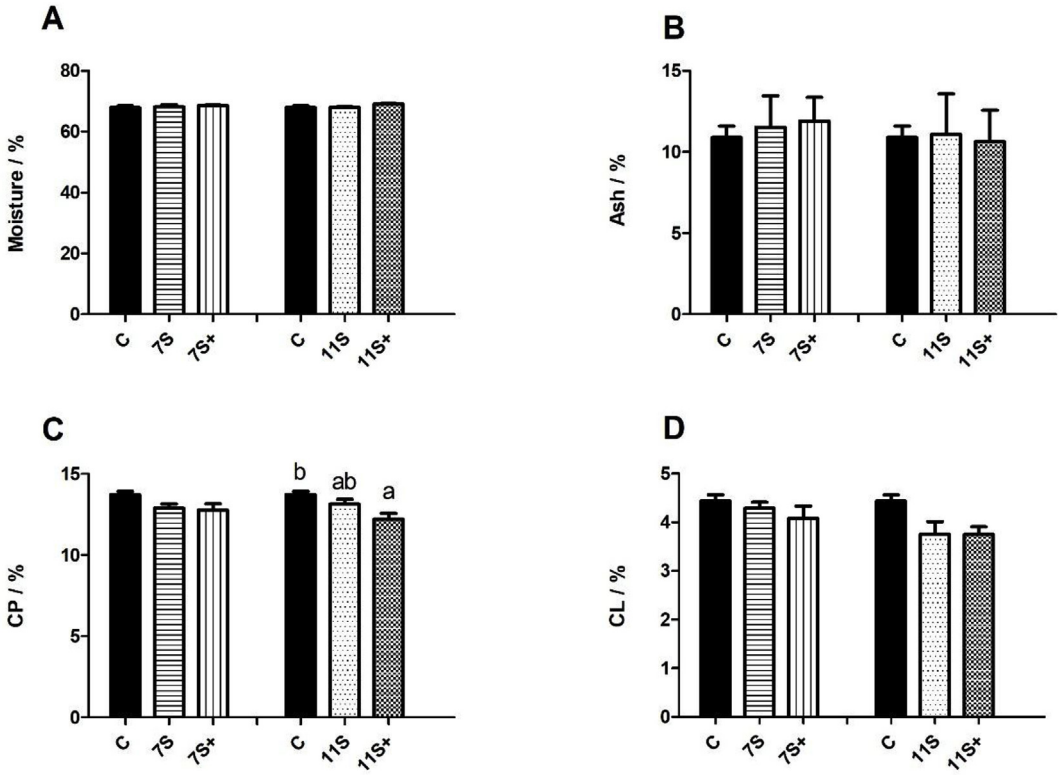

Fig. 1. Proximate composition of *Eriocheir sinensis* (% wet weight) fed different experiment diets. (A) moisture, (B) ash, (C) crude protein and (D) crude lipid. The β-conglycinin treatments (7S, 7S+) and glycinin treatments (11S, 11S+) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA, USA). Then, the purified PCR product was subjected to Illumina-based high-throughput sequencing (Personal Biotechnology Co., Ltd., Shanghai, China). The sequences obtained in this paper are available in SRA with the accession number SRP144732.

2.10. Statistical analysis

Sequences were analyzed using CL community™ software (ChunLab). The data were filtered for noisy sequences, checked for the presence of chimeras, and clustered with a threshold of 97% sequence similarity. To determine the level of sequencing depth, rarefaction curves were performed by plotting the number of observed operational

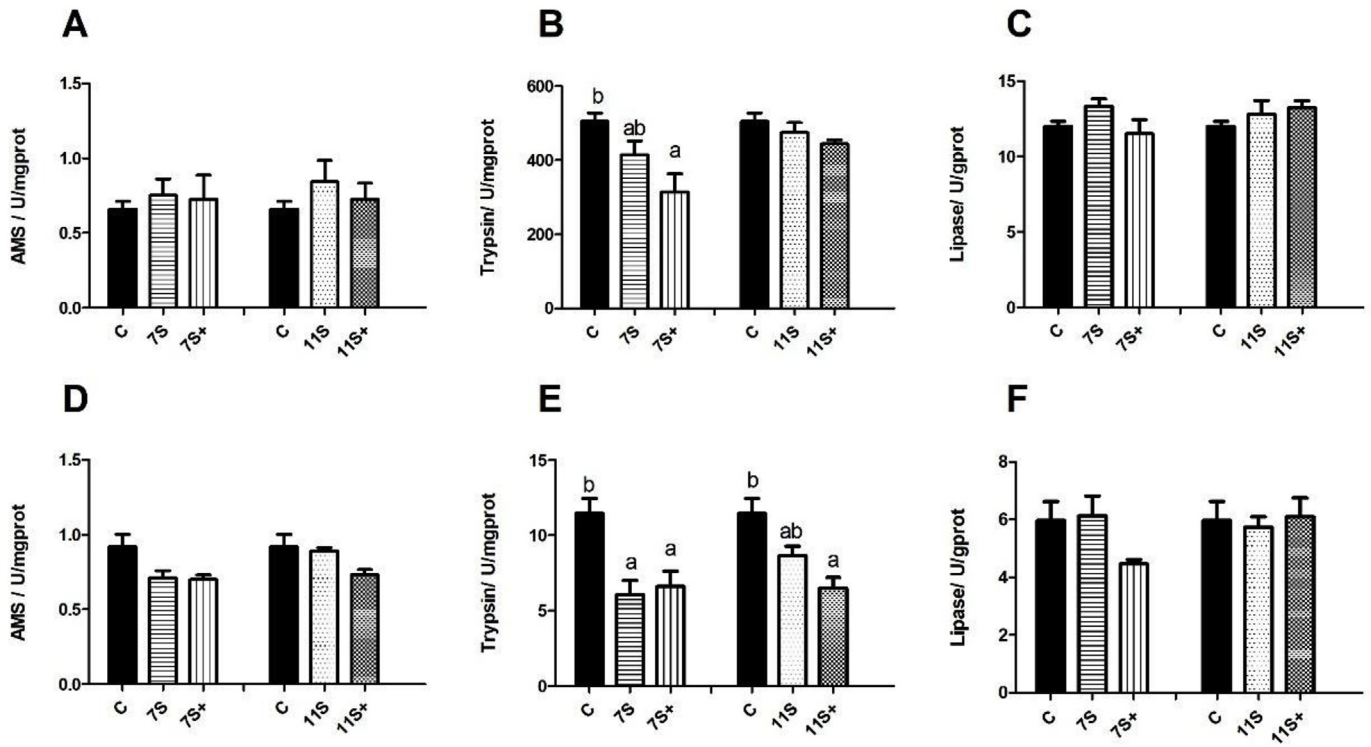

Fig. 2. The activity of amylase (A, D), trypsin (B, E) and lipase (C, F) of *Eriocheir sinensis* hepatopancreas (A–C) and intestine (D–F) fed the different experimental diets. The β-conglycinin treatments (7S, 7S +) and glycinin treatments (11S, 11S +) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

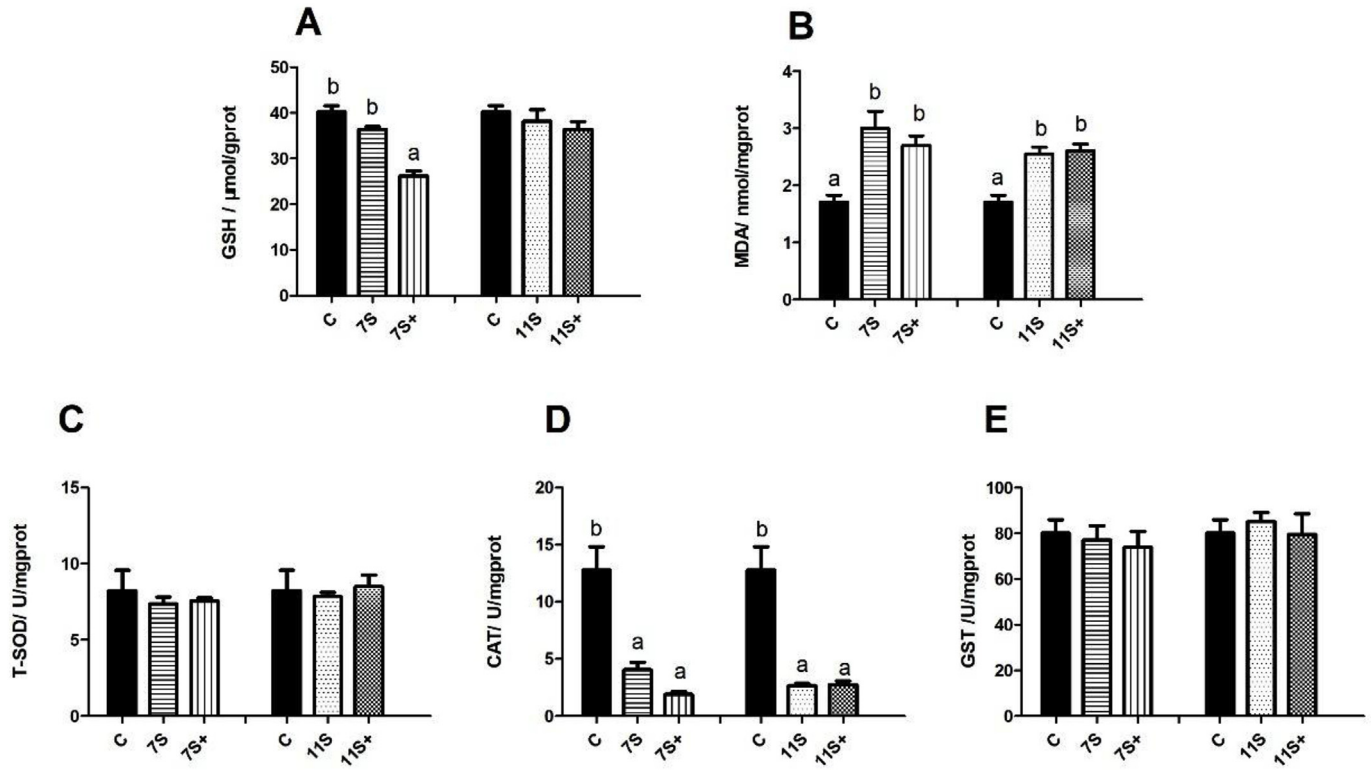

Fig. 3. The content of glutathione (A) and malondialdehyde (B), and activity of total superoxide dismutase (C), catalase(D), glutathione transferase(E) of *Eriocheir sinensis* intestine fed the different experimental diets. The β-conglycinin treatments (7S, 7S +) and glycinin treatments (11S, 11S +) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

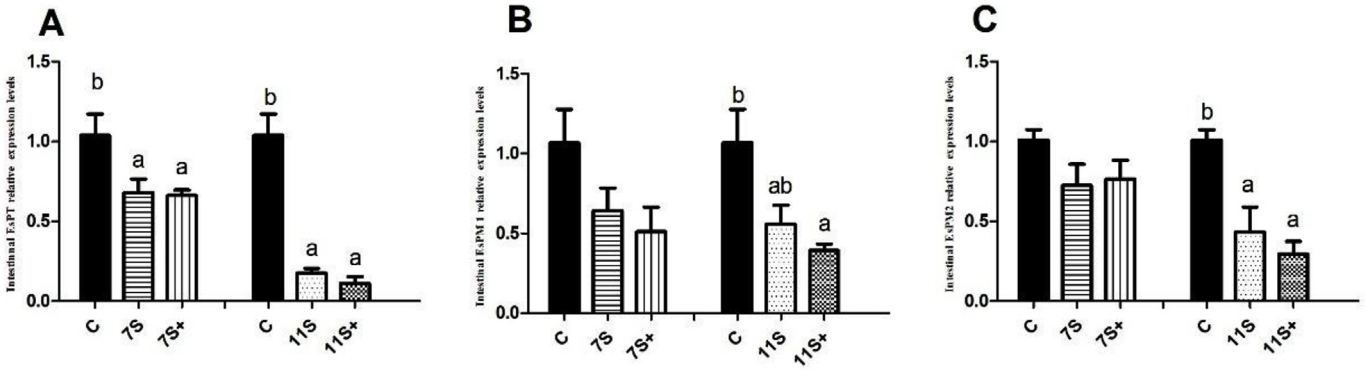

Fig. 4. Relative mRNA expression levels of the peritrophics-like and peritrophics in the intestinal tract exposed to different treatment diets. (A) EsPT, (B) EsPM1 and (C) EsPM2. The β-conglycinin treatments (7S, 7S+) and glycinin treatments (11S, 11S+) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

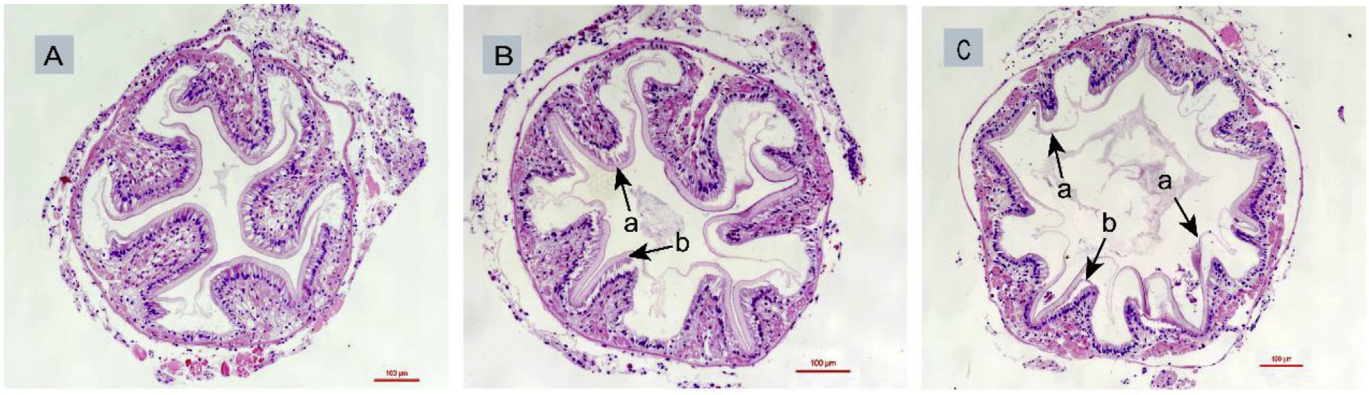

Fig. 5. Photomicrographs of the intestinal tract cross-cutting from *E. sinensis* exposed to different treatment diets with hematoxylin and eosin staining to show the changes in the intestinal folds and peritrophic membrane. Histological comparisons (A–C) on the difference in the hind-gut among the control, 70 g/kg β-conglycinin and 80 g/kg glycinin. Arrows show the pathological changes. Magnification was 100 ×, and the scale represents 100 μm. Arrow a shows the peritrophic membrane detached from the intestinal epithelial cells and folds; arrow b shows partial impairment of intestinal epithelial cells.

taxonomic units (OTU) against the number of sequences. Taxonomic richness and diversity estimators were determined in Mothur. In addition, alpha-diversity was determined to assess community diversity and it was analyzed using ACE, Chao1, Shannon-Wiener and Simpson indexes. The abundance of these OTUs were significantly different for 7S or 11S treatment compared with the control using Student's t-test. A Venn diagram was generated to represent the number of unique and shared species among percentages and groups of OTUs. The normalized abundance was exhibited by heatmap.

All statistical analyses were carried out using the SPSS version 22.0 software package. All data were subjected normality test and homogeneity of variance by using Shapiro–Wilk and Levene's equal variance tests, respectively. If the data conforms to the normal distribution, the β-conglycinin treatments (7S, 7S+) and glycinin treatments (11S, 11S+) were compared with the control (C) respectively using one-way analysis of variance (ANOVA). All data were shown as means ± standard error (means ± SE) unless otherwise indicated. Significant differences among means were determined by the Tukey's multiple range test. The value of $P < 0.05$ and $P < 0.01$ represented for statistical significance and extreme significance, respectively.

3. Results

3.1. Growth performance and survival

The results showed that the presence of β-conglycinin or glycinin in the diets could significantly reduce crab survival ($P < 0.05$). In addition, dietary inclusion of either β-conglycinin or glycinin had a significantly negative effect on final weight, weight gain and SGR of crabs ($P < 0.05$). The final weight, weight gain and SGR of crabs fed diet 7S + was significantly lower than those fed diet 7S+ ($P < 0.05$, Table 4).

3.2. Whole-body proximate composition

There were no significant differences in moisture, crude lipid and ash in both β-conglycinin and glycinin treatments. Crude protein of crabs fed diet 11S + was significantly lower than those fed the control diet ($P < 0.01$, Fig. 1).

3.3. Digestive Enzymes activities

The trypsin activity in the hepatopancreas of crabs fed 140 g/kg β-conglycinin was significantly lower than those fed the control diet ($P < 0.05$). In addition, crabs fed β-conglycinin or glycinin showed significantly lower trypsin activities in the intestine ($P < 0.05$) except for the 11S group. No significant difference was found in lipase and amylase in the hepatopancreas and intestine in both β-conglycinin and glycinin treatments ($P > 0.05$, Fig. 2).

3.4. Intestine antioxidant capacity

The diet with β-conglycinin or glycinin induced significantly higher MDA in the intestine than that in the control ($P < 0.05$), whereas CAT activities showed the opposite pattern. The GSH content of crabs fed 140 g/kg β-conglycinin was significantly lower than that in the control

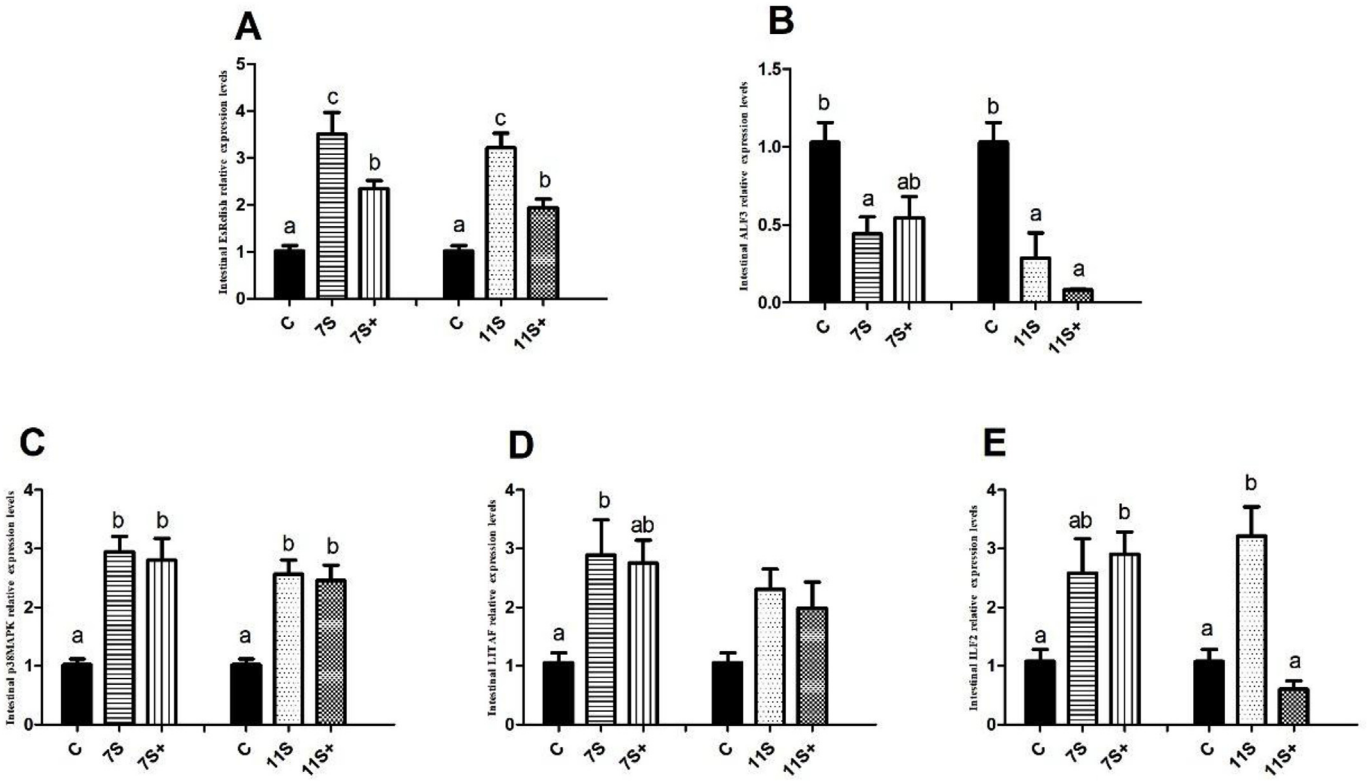

Fig. 6. Relative mRNA expression levels of the four inflammatory factors in the intestinal tract exposed to different treatment diets. (A) EsRelish, (B) ALF3, (C) p38MAPK, (D) LITAF and (E) ILF2. The β-conglycinin treatments (7S, 7S +) and glycinin treatments (11S, 11S +) compared with the control (C) respectively using one-way analysis of variance (ANOVA). Dissimilar letters show significant difference ($P < 0.05$).

($P < 0.05$). There were no significant differences in CAT and GST activities in both β-conglycinin and glycinin treatments ($P > 0.05$, Fig. 3).

3.5. Morphology of the peritrophic membrane

Compared with the control, the 80 g/kg and 160 g/kg glycinin groups significantly down-regulated the gene expression of *EsPT* and *EsPM2* in the intestine ($P < 0.05$). Besides, 160 g/kg glycinin significantly down-regulated the mRNA levels of *EsPM1* in the intestine. The 70 g/kg and 140 g/kg β-conglycinin diets significantly down-regulated the expression of *EsPT* ($P < 0.05$), whereas there was no significant change in the expression of *EsPM1* and *EsPM2* genes (Fig. 4).

The intestinal peritrophic membrane in the control closely covered the gut lumen surface, and the mucosal fold was composed of tissues with columnar epithelia (Fig. 5-A). There were spaces between the peritrophic membrane and mucosal folds in the crab fed 70 g/kg β-conglycinin, and part of the intestinal epithelial cells were disassociated from the mucosal folds (Fig. 5-B). The peritrophic membrane of the crab fed 80 g/kg glycinin was completely separated from the mucosal folds, and the partial intestinal epithelial cells were severely damaged compared with those in the 70 g/kg β-conglycinin group (Fig. 5-C).

3.6. Gene expression of EsRelish, ALF3, p38MAPK, LITAF, ILF2

The relative expressions of *EsRelish* were increased by both doses of β-conglycinin and 80 g/kg glycinin ($P < 0.05$, Fig. 6A). However, compared with the control, the lower levels of *ALF3* mRNA were found in crabs fed 70 g/kg β-conglycinin and two levels of glycinin ($P < 0.05$, Fig. 6B). The *p38MAPK* expression was significantly up-regulated by β-conglycinin or glycinin ($P < 0.05$, Fig. 6C). The mRNA expression of *LITAF* in crabs fed 70 g/kg β-conglycinin was significantly higher than that in the control ($P < 0.05$, Fig. 6D), but the *ILF2* expression was significantly higher in crabs fed 140 g/kg β-conglycinin and 80 g/kg glycinin than in the control ($P < 0.05$, Fig. 6E).

3.7. Intestinal microbiota analysis

A total of 330 231 high-quality sequences were produced in this study with an average of 36 692 sequences per sample (33 746–40 153 sequences). The obtained sequences were analyzed and grouped into different taxonomic units of bacteria. The top 20 most abundant OTUs at the genus level as inferred by *GraPhlAn* are shown in the cladogram of intestinal microbiota with *Proteobacteria* being the largest genus among the top ten OTUs (Fig. 7-a). The number of OTUs, the estimators of community richness (Ace and Chao) and diversity (Shannon and Simpson) are shown in Table 3. The results showed that no significant differences were found in these four parameters ($P > 0.05$). However, more than 97% of similar sequences were clustered into OTUs, and 851 OTUs were coincided with all the crab treatments (Fig. 7b), accounting for 93.71%, 94.57% and 90.79% of the sequences from the control, 70 g/kg β-conglycinin, and 80 g/kg glycinin groups, respectively. When compared with the control, 411 OTUs and 581 OTUs were different in the 70 g/kg β-conglycinin and 80 g/kg glycinin groups, and 331 of these OTUs were commonly found in both β-conglycinin and glycinin groups.

At the phylum level, the crabs fed both 70 g/kg β-conglycinin and 80 g/kg glycinin diets increased the abundance of Actinobacteria ($P < 0.05$) compared with the control. Besides, there was more Proteobacteria and less Bacteroidetes in the crabs fed 80 g/kg glycinin than in the control ($P < 0.05$, Fig. 8). At the genus level, *Ochrobactrum*, *Burkholderia* and *Pseudomonas* were more abundant in the 70 g/kg β-conglycinin and 80 g/kg glycinin groups than in the control ($P < 0.05$, Fig. 7-c and 9). In addition, the abundances of *Vibrio* and *Bacillus* were higher in the 80 g/kg glycinin group than in the control ($P < 0.05$, Fig. 9-b), but the abundance of *Dysgonomonas* showed an opposite

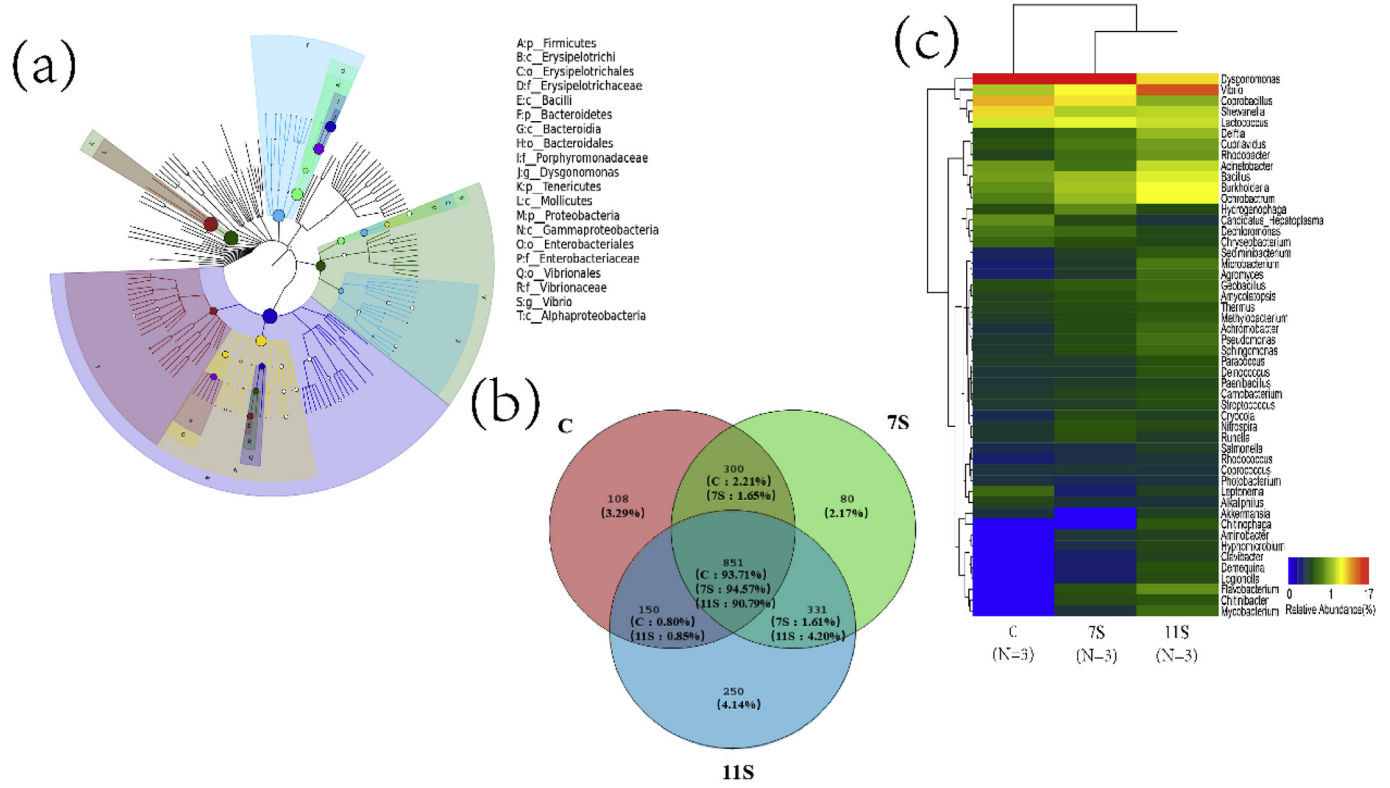

Fig. 7. (a) Cladogram of the intestinal microbiota that are the top 20 most abundant as inferred by GraPhlAn at the genus level. Node size is proportional to the average abundance; color indicates relative concentration of the clusters. (b) Venn diagrams demonstrating the distribution of the OTUs shared by *Eriocheir sinensis* in the three groups: control (C) and 70 g/kg β-conglycinin (7S) and 80 g/kg glycinin (11S). OTU numbers are generated from subsets of each group. The percentage indicates the ratio of correlated OTUs in the total sequences of each group. (c) Heatmap of the abundance of *E. sinensis* intestinal bacteria at the genus level at different experiment groups. Phylogenetic positions are projected by the OTUs, and the taxa of OTUs are listed on the right. Color intensity indicates the relative enrichment of OTUs. Control (C), 70 g/kg β-conglycinin (7S) and 80 g/kg glycinin (11S). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pattern.

4. Discussion

In the present study, crabs fed the diet with glycinin or β-conglycinin showed lower weight gain and specific growth rate than in the control, and the increase of glycinin or β-conglycinin concentrations further suppressed growth performance, which is supported by the findings in other studies on piglets and fish [5,6,25]. Presumably the reason is that glycinin and β-conglycinin in the diet interfere gastric digestion [26], and suppress the development of gastric glands in crabs. Glycinin and β-conglycinin could not be decomposed into small molecules in the digestive tract and cause allergic reaction in digestive tract, resulting in poor digestibility. Similarly, the replacement of fishmeal by > 41% soy protein in the diet could inhibit the growth performance of *L. vannamei*, though survival was not influenced [27]. Besides, no significant difference in survival was found in other similar studies on turbot and grass carp [5,6,8,28]. In this study, the survival of the crabs fed diets containing supplemented antinutritional substances was reduced significantly compared with the control.

Previous studies have shown that trypsin activity in the intestine decreases in Atlantic salmon (*Salmo salar* L) fed the 300 g soybean meal/kg diet compared to fish fed a fish meal diet [29]. Similarly, soybean meal decreases the activity of protease in tilapia, and β-conglycinin significantly decreases the activities of chymotrypsin, trypsin and lipase in the intestine of Jian carp [30]. In the present study, 140 g/kg β-conglycinin reduced the activity of trypsin in the hepatopancreas and intestine, but 160 g/kg glycinin only decreased the activity of trypsin in the intestine. The mechanism concerning the effects of glycinin or β-conglycinin on the digestive enzyme activity is not clear, but this study shows growth reduction and damage of digestive tract by β-conglycinin supplementation, which is similar to the report on tilapia [30]. The different effects of β-conglycinin and glycinin on intestinal digestive enzymes in crabs could be related to the difference of their molecular structure [31]. Glycinin has a large amount of -SH groups, and this unique topological structure enables glycinin to form a more stable and turbid gel than β-conglycinin [32]. In addition, turbot fed the diet containing 120 g glycinin/kg showed significantly lower apparent digestibility coefficients on dry matter and protein in the diet [28]. In vitro digestion trials have also demonstrated that glycinin is hardly hydrolyzed by trypsin, because of the presence of its basic subunits [33]. In this study, the body crude protein of the crabs fed 160 g glycinin/kg significantly decreased, suggesting that a higher doses of glycinin can inhibit the deposition of protein in the body.

In mammalian studies, oral administration of glycinin or β-conglycinin was able to cause hypersensitivity [34,35] and then increased permeability of mucosal edema along with crypt cell hyperplasia and shrunken intestinal villi [36]. Glycinin increased infiltration of mixed leucocytes in the distal intestine of turbot [28]. The present study shows that both glycinin and β-conglycinin damaged intestinal epithelial cells and shrank intestinal folds. In addition, our results demonstrate that the peritrophic membrane was separated from intestinal folds by the addition of dietary antigen proteins, coinciding with a significant decrease of *EsPT*, *EsPM1* and *EsPM2* mRNA expressions. The peritrophic membrane has the similar functions with goblet cells [9], which coats the epithelium tightly to improve digestion, provide mechanical and chemical protection [37]. Besides, the impact of glycinin treatment groups decreased more seriously than that in the β-conglycinin groups. *EsPT*, as

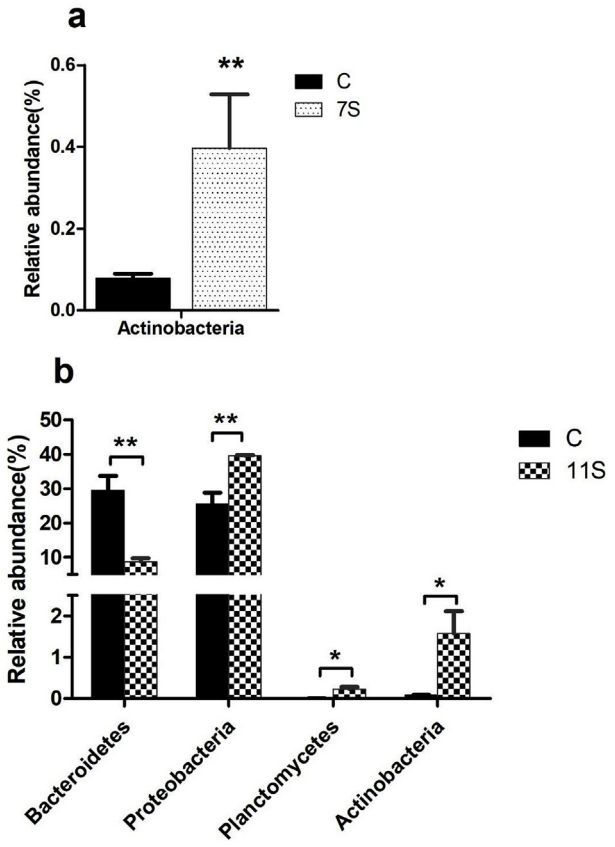

Fig. 8. Comparisons of the relative abundance of the major bacteria in the *E. sinensis* exposed to the control(C), 70 g/kg β-conglycinin (7S) and 80 g/kg glycinin (11S) at the phylum level. The LSD test was used to assess the significant differences of each phylum. Asterisk (*) represents a significant difference of $P < 0.05$ between groups. Double asterisks (**) represent a significant difference of $P < 0.01$ between groups.

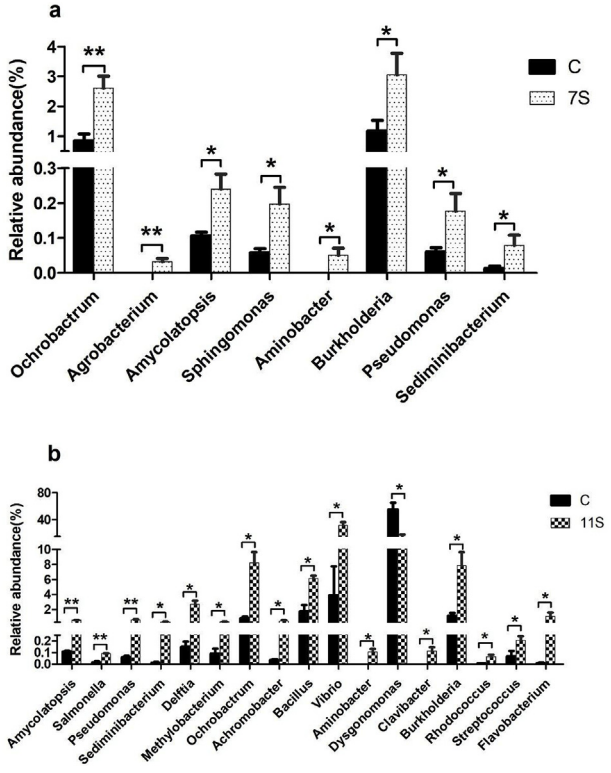

Fig. 9. Comparisons of the relative abundance of the major bacteria in the *E. sinensis* exposed to the control(C), 70 g/kg β-conglycinin (7S) and 80 g/kg glycinin (11S) at the genus level. The LSD test was used to assess the significant differences of each phylum. Asterisk (*) represents a significant difference of $P < 0.05$ between groups. Double asterisks (**) represent a significant difference of $P < 0.01$ between groups.

a peritrophin-like gene, is involved in antibacterial innate immune defense [38]. Therefore, glycinin and β-conglycinin could damage the intestinal peritrophic membrane structure of *E. sinensis*.

The disruption of intestinal peritrophic membrane integrity by glycinin and β-conglycinin may cause inflammation [5,6]. Studies on fish have demonstrated that soybean antigen protein is able to cause inflammation of the hind-gut [39,40]. It is well known that the activation of NF-κB, e.g. *EsRelish*, which is a member of the NF-κB family, may increase pro-inflammatory cytokines and suppress anti-inflammatory cytokines gene expression [41]. Moreover, the activation of *p38MAPK* may also increase the production of pro-inflammatory cytokines, such as IL-6 and TNF-α [42]. In the current study, the expressions of *EsRelish* and *p38MAPK* were also increased by glycinin or β-conglycinin. The expression of *LITAF* and *ILF2* genes, which are related to the inflammatory response and the pivotal transcription factors of TNF-α and IL-2 [43,44], were also increased by glycinin or β-conglycinin in this study. According to the above data, the inclusion of glycinin or β-conglycinin could induce the intestinal inflammation in *E. sinensis*. *ALF3* is a kind of antimicrobial peptides, and strongly exhibits antibacterial activity against Gram-negative R-type bacteria [45]. Previous studies have indicated that the expression of *ALF3* in haemocytes of *E. sinensis* increased in several hours after bacterial challenge [45,46]. The lower expression of *ALF3* indicates that the innate immunity of the intestinal tract in *E. sinensis* decreased after a long-term antigen protein stress, which supports the intestine injury found in this study.

Intestinal inflammation may lead to excessive ROS production and provoke a subsequent oxidative damage [10]. Lipid peroxidation was presented due to excessive ROS in cells by oxidative damage, while MDA is a representative of the end product of lipid peroxidation [47]. The present results show that dietary glycinin or β-conglycinin significantly increased the intestinal MDA, which is similar to the results found in Jian carp [5,6]. It is well known that oxidative damage is usually along with the reduction of non-enzymatic antioxidant compounds and the inhibition of antioxidant activity [48,49]. Surprisingly, the current study found that the content of GSH in crabs fed 140 g/kg β-conglycinin reduced significantly, and the CAT activities in the crabs fed glycinin or β-conglycinin diets also reduced significantly compared with the control. As the increased GSH content and CAT activity could scavenge excess ROS [50], crabs may consume a large amount of GSH and antioxidant enzyme (CAT) to cope with oxidative damage by glycinin and β-conglycinin.

The intestinal microbiota are essential for maintaining the integrity of mucosal structure and the function in the immune system [51]. It has been demonstrated that intestinal microbiota are affected by the environment, feed composition, developmental stage of the host [52]. Diet composition and ingredients can shape the structure of an intestinal microbial community, such as protein source, lipid source, sucrose and glucose [17,53,54]. The current study also showed that the microbial populations were affected by glycinin and β-conglycinin in the crabs. Proteobacteria accounted for a large majority of bacteria in every treatment. Similar results were also found in *Penaeus notialis* and *Carassius gibelio* [24,55,56]. At the phylum level, the abundance of Actinobacteria in both 80 g/kg glycinin and 70 g/kg β-conglycinin groups was significantly higher than those in the control. A previous study showed that the Actinobacteria were much more abundant in herbivorous fish than in other fish [57]. Therefore, the high abundance of Actinobacteria in crabs may indicate an adaptation of the crabs to plant protein in the diet. In addition, the intestine in the 80 g/kg glycinin group had higher Proteobacteria but lower Bacteroidetes

abundance than those in the control. Because Proteobacteria includes many pathogenic bacteria, a continuous enrichment of Proteobacteria can stand for the health state in the host or an imbalanced structure of intestinal microbia [58]. Bacteroides can break down polysaccharides, metabolize carbohydrates [59] and play a pivotal role in the host intestine during food digestion from complex to simple molecules. Thus, these results demonstrate that glycinin may probably change the balance of the microbiome community, and reduce the ability to decompose sugar in the *E. sinensis* intestine.

Ochrobactrum is a common soil α-proteobacterium that colonizes on a wide range of organisms, and is regarded as a potentially opportunistic pathogen in human [60]. Though few cases about this bacterium were reported in aquaculture, it has a potential risk to infect aquatic animals [61]. Similarly, *Burkholderia* is a group of pathogenic bacteria and can infect insects and human [62,63]. The *B. pseudomallei* is a Gram-negative bacterium endemic in tropical and subtropical regions worldwide and can result in fatal infection to humans termed melioidosis [64]. In addition, a broad survey of *Burkholderia* infection has indicated that the *Burkholderia* symbiosis is prevalent in the heteropteran insects [62]. However, the role of *Burkholderia* in aquatic animals is unclear. *Pseudomonas* is a group of common pathogen in aquaculture, such as *P. fluorescens* [65] *and P. aeruginosa* [66]. The infected fish by *P. fluorescens* or *P. aeruginosa*c show rotten spots on the tail, fins and gills and can lead to a high mortality [67,68]. A similar study reported that the *Pseudomonas* was more frequently found in grass carp *Ctenopharyngodon idella* fed a soybean meal diet compared to a casein meal diet [69]. Therefore, the increase in these pathogenic bacteria may indicate that glycinin and β-conglycinin can increase the risk of infection with disease in the *E. sinensis* intestine. Furthermore, the present study also found that the intestine in the 80 g/kg glycinin group had higher *Vibrio* but lower *Dysgonomonas* abundance than those in the control. As a typical representative of *Vibrio*, *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* are often reported to be the leading cause of disease outbreaks in *L. vannamei* aquaculture [70,71]. Thus, this finding suggests that glycinin exposure can increase the susceptibility of *E. sinensis* to *Vibrio* infection. So far, *Dysgonomonas macrotermitis* is the only species of *Dysgonomonas* that has been found in invertebrates [72]. The major function of *Dysgonomonas macrotermitis* in the hind-intestine of *Macrotermes barneyi* is to decompose lignocellulose and provide nutrition to the host [72]. Therefore, the decrease of *Dysgonomonas* indicates that glycinin exposure may reduce the ability of *E. sinensis* to take advantage for digesting plant raw materials. Many other bacteria have also exhibited significant alterations in glycinin or β-conglycinin group compared with the control, but the relationships between microbiota, intestinal inflammatory responses, and causes of microbial community alteration need further investigation.

5. Conclusion

Both dietary β-conglycinin and glycinin could induce inflammation and oxidation damage in the intestine, shape the structure of gut microbial community, and cause dysfunction of the digestive system in crabs. This may reduce the growth of crab when fishmeal is replaced by soybean meal in the diet. The number of pathogen species in the gut was higher when the crab were fed with 80 g/kg glycinin than those were fed with 70 g/kg β-conglycinin in the diet. The anti-nutritive effect in the *E. sinensis* is more pronounced to glycinin than β-conglycinin.

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