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Positive impacts of dietary prebiotic inulin on growth performance, antioxidant capacity, immunity, and intestinal microbiota of red swamp crayfish (*Procambarus clarkii*)

Qian Wang[1,3] · Yanbin Lin[1,3] · Heng Zhang[2] · Wenhao Fan[1,3] · Shengxuan Li[1,3] · Guoliang Ruan[1,2,3] · Liu Fang[1,2,3]

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Abstract
Inulin is known to be a prebiotic used in aquatic animals. However, no investigation has been conducted to evaluate its effect on freshwater crayfish. In this study, a 7-week feeding trial using diets supplemented with inulin (0.0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.0% feed) was conducted on 360 red swamp crayfish (*Procambarus clarkii*) (initial body weight 6.58 ± 0.16 g) with four parallels each group, in order to determine the effects of dietary inulin on the growth performance, antioxidant capacity, immune response, and intestinal microbiota of this crayfish. Firstly, the feeding trials showed that the survival rate and growth performance of *P. clarkii* fed 0.6% dietary inulin were significantly improved and feed conversion ratio was significantly reduced. Secondly, the antioxidant capacity of hepatopancreas was significantly improved by inulin supplementation. The crayfish fed 0.6% dietary inulin had the lowest malondialdehyde content and the highest antioxidant enzyme (T-AOC, T-SOD, GSH-PX, and CAT) activities. The addition of 0.6% and 0.8% dietary inulin significantly increased the expression levels of immune-related genes in the intestine and hepatopancreas. Moreover, high-throughput sequencing of 16S rRNA showed that 0.6% dietary inulin altered the beta diversity and composition of the intestinal microbiota, with a significant increase in the relative abundance of the *Citrobacter* spp. Meanwhile, intestinal microbial KEGG pathway analysis showed that 0.6% dietary inulin promoted metabolism, digestion, transport, circulation, and cellular processes in *P. clarkii*. This study indicated that 0.6% dietary inulin was appropriate for *P. clarkii* to improve the growth, antioxidation, immunity, and intestinal health.

Keywords Inulin · *Procambarus clarkii* · Growth performance · Antioxidation · Immunity · Intestinal microbiota

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Extended author information available on the last page of the article

Introduction

The red swamp crayfish (*Procambarus clarkii*) is a worldwide invasive species with omnivorous, rapid growth, and high fecundity, widely distributed in varieties of freshwater areas in China (Larson et al. 2017; Yi et al. 2018). Due to its tasty and nutritious meat, crayfish is nowadays one of the most popular aquatic foods, and its culture has been well developed in the last two decades and now accounts for the largest proportion of freshwater crustacean aquatic animals farmed in China (Bureau 2022). However, crayfish have shown poor growth performance and high susceptibility to pathogens in the context of ecological degradation caused by intensive farming (Huang et al. 2021; Romaire and Villagran 2010; Yu et al. 2020; Zhu et al. 2021). Moreover, the use of antibiotics and other chemical agents (e.g., disinfectants, pesticides, and herbicides) is inevitable in the rice-crayfish integrated systems, which is the dominant mode of crayfish culture (Hemamalini et al. 2022; Jimenez et al. 2003; Ma et al. 2019; Yu et al. 2018, 2017). The residues and accumulation of these agents, and the resulting pathogen's resistance, in turn cause deterioration to the aquatic ecology and impairment to the crayfish's adversity resistance (Dan et al. 2019; Hemamalini et al. 2022; Ma et al. 2019), which ultimately results in economic losses to crayfish farmers. Therefore, it will be crucial to improve the health status and growth performance of aquatic animals through an environment-friendly model.

Prebiotics are not digestible by the animal, but they have a positive effect on the host by selectively stimulating the growth or activity of one/some beneficial bacteria in the host's digestive tract (Gibson et al. 2004). Recently, prebiotics have been widely used on aquatic animals as an alternative strategy to antibiotics (Butt et al. 2021; Daniels and Hoseinifar 2014; Guerreiro et al. 2018; Ringø et al. 2010). As one of typical prebiotics, inulin is an indigestible but fermentable fructan-type plant polysaccharide, composed by fructose monomers linked via β-(2–1)-D-frutosyl fructose bonds and a glucose molecule at C2 end (Mudgil 2017; Shoaib et al. 2016).

The dietary supplementation of inulin in farmed crustaceans appears to achieve favorable effects, including enhancing survival and growth, antioxidation and immunity, and resistance to pathogens, as well as altering or improving the intestinal microflora of host. For instance, supplementations of 4–5 mg/g inulin in the diet significantly promote the expression of growth-related and immune-related marker genes and increased the weight gain and specific growth rate of *Litopenaeus vannamei* (Li et al. 2018, 2021; Zhou et al. 2020). Dietary inulin also increases superoxide dismutase, catalase, phenol oxidase, and acid phosphatase activities and reduces malondialdehyde levels in the hepatopancreas of *L. vannamei* (Zhou et al. 2020). Feeding inulin-rich diets significantly increases the levels of lactic acid bacteria in the gut and the survival rate of Indian white shrimp post-larvae (*Fenneropenaeus indicus*) (Hoseinifar et al. 2015). Dietary inulin also significantly increases the relative abundance of beneficial bacteria such as *Bacillus* and *Lactobacillus* and significantly decreases the relative abundance of harmful bacteria such as *Vibrio* in the intestine of *L. vannamei*, and significantly increases the resistance to WSSV or *V. alginolyticus* infection (Li et al. 2021; Luna-González et al. 2012). Nevertheless, some ineffective results were recorded in studies on fish and shrimp species (Bolívar Ramírez et al. 2013; Guerreiro et al. 2018; Gutiérrez-Dagnino et al. 2015; Hoseinifar et al. 2010; Luna-González et al. 2012). Besides, to date, most of the research on inulin as crustacean prebiotic has been focused on penaeid shrimps (e.g., *L. vannamei*). To the best of our knowledge, there is no report regarding the application of inulin in freshwater crayfish culture.

Accordingly, to provide a better understanding of the prebiotic effects of dietary inulin on *P. clarkii*, a 7-week of feeding experiment was conducted on this crayfish fed using diets supplemented with different levels of inulin. In this study, the effects of dietary inulin supplementation on growth performance, antioxidant capacity, immune response, and intestinal microbiota changes of *P. clarkii* were determined, and the optimal dietary supplementation in *P. clarkii* diets was investigated. Our study will provide a practical reference for the application of inulin in decapod aquatic animals and for environment-friendly feed and healthy culture of crayfish.

Materials and methods

Experimental diets

The trail diets were prepared in accordance with the nutritional requirement of *P. clarkii* (Fu et al. 2022). The six experimental diets contained 0.0% (control), 0.2%, 0.4%, 0.6%, 0.8%, and 1.0% inulin at different levels (Chongqing Jiaowang Natural Products Co., Ltd., China, purity ≥ 99%), with four parallel settings for each treatment. All ingredients were ground into powder through a 0.18-mm sieve and mixed thoroughly, then extruded into 2-mm diameter pellets in a feed compressor (NBS-150, Henan Zanxi Industrial Factory, Henan, China). The diets were placed in a desiccator at 60 °C for 12 h, then packed and stored at − 20 °C until use. The ingredients and proximate components of diets are indicated in Table 1.

Animal rearing and feeding trial

The crayfish (approximately 600 individuals) were sourced from a local farm in Gongan County, Jingzhou, Hubei, China, and were temporarily reared for 2 weeks with a basal diet that was consistent with the composition of the control diet to acclimatize them to the experimental conditions before the formal feeding trial. The handling of animals in this study followed the principles of good laboratory animal care and was approved by the Ethics Committee for the Care and Use of Laboratory Animals of Yangtze University. After acclimation, responsive and vigorous crayfish, which had intact appendages and clearly discernible grayish-black "abdominal vein" (i.e., the gut with consecutive and full contents), was considered healthy individual, and then 360 healthy crayfish with an average initial body weight (IBW) of 6.58 ± 0.16 g and an average initial body length (IBL) of 55.12 ± 0.79 mm were selected and then randomly assigned to an indoor culture system consisting of 24 round fiberglass tanks (60 cm × 40 cm × 35 cm) with 15 animals per tank. To provide habitat and reduce aggressive behavior, some polyethylene nets and PVC pipes were placed in each tank as shelters for crayfish.

Crayfish were hand-fed twice daily (9: 00 and 18: 00) at a rate of 3–4% of body weight. One hour after feeding, unconsumed feed was aspirated and dried to determine the actual feed consumption. Throughout the 7-week feeding trial, one-quarter to one-half of the water was exchanged daily and water quality was monitored and maintained

Table 1 Ingredients and proximate composition of the experimental diets with different inulin contents (expressed as % in dry matter)

Items	Dietary inulin levels					
	0.0%	0.2%	0.4%	0.6%	0.8%	1.0%
Ingredients						
Fish meal	320	320	320	320	320	320
Soybean meal	150	150	150	150	150	150
Soybean oil	25	25	25	25	25	25
Fish oil	25	25	25	25	25	25
Strong flour	130	130	130	130	130	130
Alpha starch	240	240	240	240	240	240
Soybean lecithin	10	10	10	10	10	10
Vitamin premix[a]	10	10	10	10	10	10
Mineral premix[b]	10	10	10	10	10	10
Ecdysone	2	2	2	2	2	2
Antioxidants	1	1	1	1	1	1
Choline chloride	1	1	1	1	1	1
Microcrystalline cellulose	30	28	26	24	22	20
$Ca(H_2PO_4)_2$	14	14	14	14	14	14
Betaine	2	2	2	2	2	2
Sodium alginate	30	30	30	30	30	30
Inulin	0	2	4	6	8	10
Total	1000	1000	1000	1000	1000	1000
Nutrient levels[c]						
Moisture	3.56 ± 0.02	3.55 ± 0.03	3.55 ± 0.02	3.55 ± 0.01	3.54 ± 0.03	3.58 ± 0.01
Crude protein	28.35 ± 0.43	28.16 ± 0.58	28.75 ± 0.23	28.20 ± 0.25	28.92 ± 0.33	28.43 ± 0.46
Crude lipid	11.42 ± 0.34	11.21 ± 0.26	11.58 ± 0.10	11.58 ± 0.17	11.12 ± 0.53	11.55 ± 0.03
Ash	11.14 ± 0.37	11.40 ± 0.67	11.01 ± 0.77	11.04 ± 0.08	11.19 ± 0.33	10.96 ± 0.18

[a]one kilogram of vitamin premix contained the following: VA 200 000 IU, VB_1 4 g, VB_2 8 g, VB_6 4.8 g, VB_{12} 16 mg, VD_3 1 600 000 IU, VE 16 g, VK 4 g, calcium pantothenate 16 g, folic acid 1.28 g, nicotinic acid 28 g, inositol 40 g, biotin 64 mg. [b]One kilogram of mineral premix contained the following: $MgSO_4$ 12 g, $Ca(IO_3)_2$ 9 g, KCl 36 g, Met-Cu 1.5 g, $ZnSO_4$ 10 g, $MnSO_4$ 15 g, $FeSO_4$ 1 g, Met-Co 250 mg, $NaSeO_3$ 3.6 mg. [c]Measured values

at a temperature of 23 ± 1.0 °C, dissolved oxygen ≥ 6.0 mg/L, pH of 7.2–8.5, and ammonia nitrogen ≤ 0.02 mg/L, respectively. A natural light/dark regime was used for this experiment.

Sample collection

The crayfish were fasted for 24 h at the end of the feeding trial in order to empty their intestinal tract. All crayfish in each tank were counted, measured, and weighted for calculation of survival rate (SR), final body weight (FBW), final body length (FBL), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and condition factor (CF). Subsequently, 4 crayfish were randomly selected from each tank (16 crayfish

for each dietary treatment) and anesthetized on ice. The hepatopancreases, muscles, and intestines of these 4 crayfish were dissected out on the ice, and the hepatopancreas was weighed to compute hepatosomatic index (HSI). The aforementioned samples were then collected and stored at − 80 °C for analytical determination of proximate composition (muscle), antioxidant capacity (hepatopancreas), and expression of immune-related genes (hepatopancreas and intestine). Another 4 crayfish were randomly selected from each tank and then stored at − 20 °C for whole-body proximate composition determination.

Computation of growth performance parameters

In this study, all related parameters of growth performance were calculated by using the following formulas:

$$\mathrm{SR}\ (\%) = 100 \times (\text{Final crayfish number}/\text{Initial crayfish number});$$
$$\mathrm{WG}\ (\%) = 100 \times \left[(\mathrm{FBW} - \mathrm{IBW})/\mathrm{IBW}\right];$$
$$\mathrm{SGR}\ \left(\% \cdot \mathrm{day}^{-1}\right) = 100 \times \left[(\ln\ \mathrm{FBW} - \ln\ \mathrm{IBW})/\mathrm{t}\right];$$
$$\mathrm{FCR} = 100 \times (\text{Dry feed intake}/\text{Wet weight gain of crayfish});$$
$$\mathrm{HSI}\ (\%) = 100 \times (\text{Wet weight of hepatopancreas}/\text{Wet weight of crayfish});$$
$$\mathrm{CF}\ \left(\mathrm{g}/\mathrm{cm}^3\right) = 100 \times [(\mathrm{FBW}/(\mathrm{FBL}^3)];$$

Proximate composition determination of diet and crayfish

The proximate compositions of the whole-crayfish, muscle, and diets were analyzed following the standard procedures (AOAC 2005). Briefly, the content of moisture was determined by drying at 105 °C to a constant weight. Crude protein (CP) was measured using the Kjeldahl method. Crude lipid (CL) was calculated by the Soxhlet extraction method. Ash content was determined using a muffle furnace by incinerating the sample at 550 °C.

Determination of antioxidant enzyme activities and MDA content

To the weighed hepatopancreas sample, 10 times the volume (v/w) of pre-cooled sterile 0.9% NaCl solution was added, homogenized, and centrifuged at 3500 r/min for 10 min at 4 °C. The supernatant was aspirated for the next measurement (Guo et al. 2021). Commercially available kits (Nanjing Jiancheng, China) were used to determine the total antioxidant capacity index (T-AOC), activities of total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) content, following the manufacturer's instructions (Ruan et al. 2022).

Total RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from the hepatopancreas and intestine of 4 crayfish per tank using RNAiso Plus reagent (Takara, Japan). The first-strand cDNA was synthesized by using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). The RT-qPCR analyses of anti-lipopolysaccharide factor (*ALF*), *Crustin*, and *Lectin* genes were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) by using the TB Green® Premix

Table 2 Primers used for RT-qPCR analysis of immune-related genes in this study

Primers	Sequences (5′-3′)	Genbank accession No
18S-F	CTGTGATGCCCTTAGATGTT	KX444578.1
18S-R	GCGAGGGGTAGAACATCCAA	
ALF1-F	GCGGGTTATGGAAAGATGG	KU680792.1
ALF1-R	CCTGACGAAGTCCCTGGTG	
Crustin1-F	GGCGTCTGTGGAGGATGG	GQ301201.1
Crustin1-R	GAGGTGGTTAGGGTAGTTGAGC	
C-type-lectin3-F	CTCAGGCAGAGTCGTGTCCC	JX844151.1
C-type-lectin3-R	ACCATTCACCTTCCTGTCCAA	
338F	ACTCCTACGGGAGGCAGCAG	
806R	GGACTACHVGGGTWTCTAAT	

DimerEraser™ assay kit (Takara, Japan). The 18S rRNA gene was employed as an internal control. The RT-qPCR program was set up as below: 95 °C for 30 s, and then 40 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 10 s, and fluorescent signal were detected at 72 °C. Gene relative expression levels were calculated by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The primers used in RT-qPCR are indicated in Table 2 (Zhang et al. 2020).

Intestinal microbiota analysis

A total of 12 crayfish were selected from the control group (CG) and the 0.6% inulin added group (EG) and randomly divided into three replicates of 4 crayfish each. After dissecting the crayfish on ice, whole intestines were obtained, washed in PBS twice, rapidly frozen in liquid nitrogen, and then stored at − 80 °C for microbiota analysis.

Bacterial genomic DNA was extracted using an E.Z.N.A.® Soil DNA Kit (Omega, USA). A universal primer pair 338F and 806R (Table 2) were used to amplify the highly variable V3–V4 region of the bacterial 16S rRNA gene. The PCR program was set up as below: 95 °C for 3 min, 27 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. According to the standard protocol of Majorbio Biomedical Technologies, Inc (Shanghai, China), paired-end sequencing of purified amplicons was performed using the Illumina MiSeq platform (Illumina, USA). Raw sequencing data were merged using the sliding window method and were paired using FLASH (https://ccb.jhu.edu/software/FLASH/index.shtml) according to overlapping bases. Qualified reads were clustered to generate operational taxonomic units (OTUs) according to the 97% similarity standard using the UPARSE (http://drive5.com/uparse/). Data analysis was performed on the Majorbio online platform (http://www.majorbio.com). Briefly, sparse curves were created to determine if the sequencing depth was sufficient to cover the expected number of OTUs. Alpha diversity indices (Chao1, ACE, Simpson, Shannon and Coverage index), beta diversity analysis (PCoA), and Venn diagrams were all performed at the OTU level. Metagenomic function prediction was based on level 1 and level 2 functional categories from the KEGG pathway database by using software PICRUSt2 (Community Phylogenetic Survey by Reconstruction of Unobserved States).

Statistical analysis

Values were expressed as mean ± standard deviation (SD). One-way ANOVA was performed using SPSS 24.0, followed by Tukey's multiple comparison test to analyze differences between groups. $P < 0.05$ was considered to be statistically significant.

Results

Changes in growth performance

Since the crayfish had adapted to the experimental environment and diet during the 2-week acclimatization period, they were generally in good vigor and health during the formal trials, and the experimental diets supplemented with different doses of inulin were well accepted by different groups of crayfish. The growth performance parameters of crayfish during the 7-week feeding trial are indicated in Table 3. Survival rate (SR) was higher in all inulin-supplemented groups than in the control group, with the 0.6% inulin group having the highest SR (no mortality) during the trial ($P < 0.05$). Crayfish fed different levels of inulin exhibited higher WG/SGR and lower FCR compared to the control group ($P < 0.05$), with the 0.6% inulin group showing the best growth performance ($P < 0.05$). HSI increased with increasing inulin supplementation and was significantly higher in the group fed 0.6–1.0% inulin compared with the control group ($P < 0.05$), but CF did not differ among the groups ($P > 0.05$).

Figure 1 illustrates that the relationship between the growth data, specifically FBW and SGR, and dietary inulin levels was best represented by the broken-line model, and it was determined that the optimal dietary inulin level (ODIL) for crayfish growth was 0.69% or 0.70%.

Table 3 Growth performance and morphology parameters of *P. clarkii* with different levels of inulin in the diet

Items	Dietary inulin levels					
	0.0%	0.2%	0.4%	0.6%	0.8%	1.0%
SR (%)	70.00 ± 15.28^{a}	90.00 ± 10.00^{ab}	87.50 ± 7.50^{ab}	100 ± 0.00^{b}	93.33 ± 6.67^{ab}	85.00 ± 8.66^{ab}
IBL (mm)	54.30 ± 0.49	54.17 ± 0.63	55.91 ± 0.48	54.86 ± 0.65	55.51 ± 0.53	55.94 ± 0.40
FBL (mm)	67.50 ± 1.30^{a}	67.39 ± 1.13^{a}	69.60 ± 0.74^{ab}	73.78 ± 1.34^{c}	74.08 ± 1.91^{c}	72.31 ± 1.06^{bc}
IBW (g)	6.30 ± 0.15	6.59 ± 0.21	6.79 ± 0.13	6.56 ± 0.14	6.66 ± 0.09	6.58 ± 0.17
FBW (g)	13.02 ± 0.59^{a}	14.41 ± 0.39^{b}	14.44 ± 0.30^{b}	19.67 ± 1.13^{c}	19.09 ± 0.28^{c}	16.04 ± 0.75^{d}
WG (%)	106.61 ± 1.17^{a}	120.22 ± 4.55^{b}	124.79 ± 3.57^{b}	207.09 ± 8.78^{c}	202.24 ± 4.26^{c}	153.76 ± 12.64^{d}
SGR (% day^{-1})	1.48 ± 0.01^{a}	1.61 ± 0.04^{b}	1.65 ± 0.04^{b}	2.29 ± 0.06^{c}	2.26 ± 0.03^{c}	1.90 ± 0.10^{d}
FCR	1.88 ± 0.04^{a}	1.78 ± 0.04^{b}	1.71 ± 0.03^{bc}	1.45 ± 0.07^{d}	1.58 ± 0.05^{e}	1.64 ± 0.06^{ce}
HSI (%)	5.55 ± 0.62^{a}	5.69 ± 0.14^{a}	6.25 ± 0.14^{a}	8.76 ± 0.15^{b}	8.37 ± 0.45^{b}	9.12 ± 0.24^{b}
CF (g/cm^3)	4.28 ± 0.04	4.26 ± 0.08	4.40 ± 0.07	4.29 ± 0.07	4.26 ± 0.04	4.38 ± 0.03

SR, survival rate; *IBL*, initial body length; *FBL*, final body length; *IBW*, initial body weight; *FBW*, final body weight; *WG*, weight gain; *SGR*, specific growth rate; *FCR*, feed conversion ratio; *HSI*, hepatosomatic index; *CF*, condition factor. Values are presented as means ± standard deviation (SD; $n = 3$). Data not sharing a common superscript within the same row are significantly different ($P < 0.05$), while data within the same row without any superscript are not different

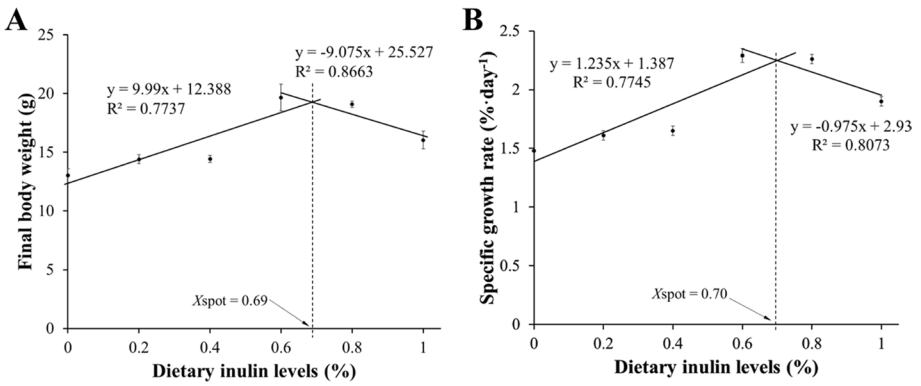

Fig. 1 Broken-line analysis of the relationship between dietary inulin supplemental level and growth performance of *P. clarkii*. **A** The regression analysis between final body weight and dietary inulin level; **B** the regression analysis between specific growth rate and dietary inulin level

Changes in whole-crayfish and muscle composition

The inulin supplementation significantly altered proximate compositions of muscle and whole body, except for moisture (Table 4). The muscle CP content of all the inulin groups was significantly higher than that of the control group ($P < 0.05$), and there was no significant difference in CP content among the inulin groups ($P > 0.05$). With the increase of inulin addition, the CL and ash contents of muscle first increased and then decreased, and the CL content of muscles in the 0.2–0.4% inulin group increased significantly compared with the control or 1.0% inulin groups ($P < 0.05$), and the ash content of muscles in the 0.4% inulin group was significantly higher than any of the other groups ($P < 0.05$). The CP and CL contents of the whole crayfish gradually increased with the increase of dietary inulin and reached the maximum in the 1.0% inulin group, respectively ($P < 0.05$). The addition of dietary inulin significantly reduced the ash content of the whole body ($P < 0.05$) and tended to decrease with the increase of inulin addition except for the 1.0% inulin group.

Changes in oxidative damage and antioxidant indicators

Dietary supplement with inulin significantly altered the antioxidant capacity of crayfish. Compared with the control, all crayfish in the inulin-added groups exhibited significantly higher antioxidant enzyme activities and significantly lower MDA contents, respectively (Fig. 2). The T-AOC (Fig. 2A) and GSH-PX (Fig. 2D) activities in the hepatopancreas of the 0.4% inulin group were the highest among all groups ($P < 0.05$). Correspondingly, the predicted ODIL was 0.52% (Fig. 2F) and 0.59% (Fig. 2I), respectively, based on the fitted quadratic curves. Similarly, the 0.6% inulin group showed the highest activity of T-SOD (Fig. 2B) and CAT (Fig. 2C) ($P < 0.05$) with corresponding ODIL of 0.74% (Fig. 2G) and 0.72% (Fig. 2H), respectively. With the increase of inulin supplementation, the MDA content increased and then decreased, showing the lowest value in the 0.6% inulin group (Fig. 2E) ($P < 0.05$), with a corresponding ODIL of 0.64% (Fig. 2J).

Table 4 Muscle and whole-body proximate compositions of *P. clarkii* with different levels of inulin in the diet

Groups	Muscle				Whole body			
	Moisture	CP	CL	Ash	Moisture	CP	CL	Ash
0.0%	80.85 ± 1.03	14.11 ± 0.15^{a}	1.61 ± 0.01^{ab}	1.10 ± 0.05^{a}	70.43 ± 0.67	12.50 ± 0.30^{a}	3.20 ± 0.06^{a}	11.09 ± 0.10^{e}
0.2%	80.54 ± 0.76	16.40 ± 0.37^{b}	2.01 ± 0.09^{d}	1.34 ± 0.01^{c}	70.89 ± 1.00	13.61 ± 0.74^{ab}	3.21 ± 0.05^{a}	10.11 ± 0.25^{d}
0.4%	80.57 ± 0.72	16.54 ± 0.62^{b}	1.92 ± 0.08^{cd}	1.41 ± 0.01^{d}	72.11 ± 1.20	12.78 ± 0.61^{a}	3.36 ± 0.19^{ab}	9.26 ± 0.19^{c}
0.6%	81.11 ± 0.93	16.42 ± 0.07^{b}	1.78 ± 0.05^{bc}	1.33 ± 0.01^{c}	72.41 ± 0.91	12.94 ± 0.76^{ab}	3.37 ± 0.14^{ab}	8.39 ± 0.14^{b}
0.8%	80.50 ± 0.89	16.66 ± 0.08^{b}	1.79 ± 0.02^{bc}	1.29 ± 0.01^{bc}	73.11 ± 0.84	13.49 ± 0.19^{b}	3.36 ± 0.12^{ab}	8.04 ± 0.13^{a}
1.0%	81.35 ± 0.92	16.24 ± 0.20^{b}	1.58 ± 0.09^{a}	1.24 ± 0.02^{b}	72.13 ± 1.68	13.88 ± 0.20^{b}	3.41 ± 0.08^{b}	9.41 ± 0.14^{c}

CP, crude protein; *CL*, crude lipid. Values are presented as means ± standard deviation (SD; $n = 3$). Data not sharing a common superscript within the same column are significantly different ($P < 0.05$), while data within the same column without any superscript are not different

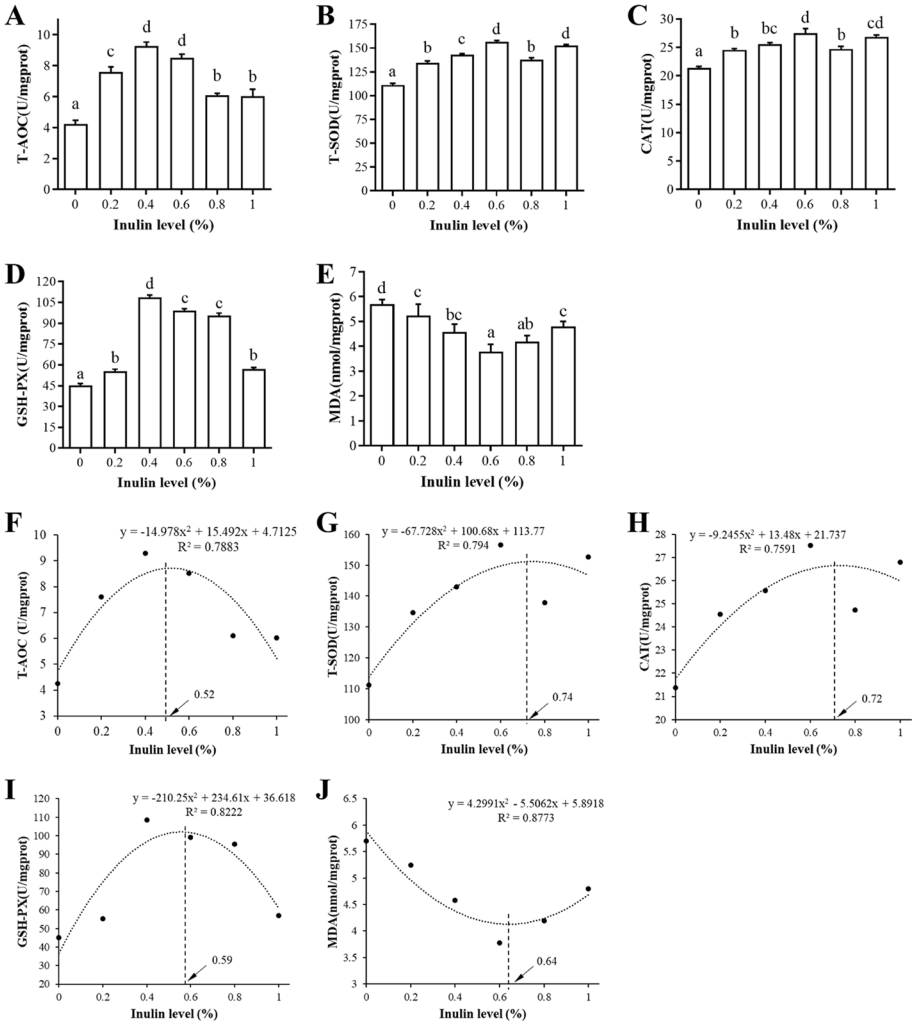

Fig. 2 Effects of different levels of dietary inulin on antioxidant enzyme activities (**A**–**D**) and MDA contents (**E**) in hepatopancreas and their optimal values (**G**–**J**) of *P. clarkii* based on the fitted quadratic curves. Different letters above bars indicated significant differences ($P < 0.05$)

Changes in the expression of immune-related genes

As shown in Fig. 3, with the increased addition of dietary inulin, the relative expression levels of three immune-related genes, except for intestinal *Lectin* and hepatopancreas *ALF*, were significantly higher than that of the control group ($P < 0.05$), and showed a significant trend of ascending and then descending. For *ALF* in the intestine (Fig. 3A) and hepatopancreas (Fig. 3D), *Crustin* in the hepatopancreas (Fig. 3E), and *Lectin* in the intestine (Fig. 3C), the relative expressions reached the highest level among all the treatments when the dietary inulin content was 0.6% ($P < 0.05$). Similarly, for *Crustin* in the intestine (Fig. 3B) and *Lectin*

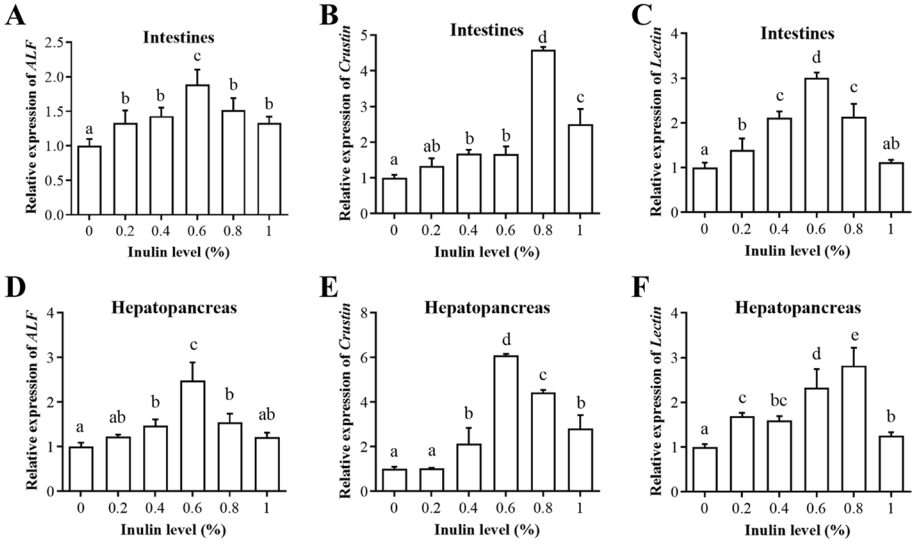

Fig. 3 Effects of different levels of dietary inulin on the relative expression of immune-related genes in the intestine (**A**–**C**) and hepatopancreas (**D**–**F**) of *P. clarkii*. Different letters above bars indicate significant differences ($P < 0.05$)

in the hepatopancreas (Fig. 3F), the relative expressions reached the highest level when the dietary inulin content increased to 0.8% ($P < 0.05$).

Changes in alpha and beta diversity of intestinal microbiota

Through Illumina Miseq high-throughput sequencing, 72,014 ± 2411 and 72,029 ± 1101 high quality reads were obtained from the CG group (control diet) and EG group (0.6% inulin addition), respectively, with a sample coverage of more than 99.90% (Table 5). The rarefaction curves indicated that the sequencing was comprehensive and representative of the true species composition in the samples (Fig. 4A).

The alpha diversity indices of the intestinal microbiota are indicated in Table 5. Compared to the CG group, the EG group showed a slight decrease in Shannon and Simpson indices, but a slight increase in Chao and ACE indices. Overall, the diversity and richness of microbial

Table 5 Alpha diversity indices in *P. clarkii* intestines of control condition (CG) and inulin treatment (EG)

Groups	OTUs	Diversity index		Coverage (%)	Richness index	
		Shannon	Simpson		Chao1	Ace
CG	290.67 ± 17.62	2.48 ± 0.44	0.07 ± 0.03	99.96 ± 0.02	304.42 ± 22.12	304.31 ± 24.42
EG	310.67 ± 44.66	2.88 ± 0.56	0.15 ± 0.11	99.95 ± 0.01	327.01 ± 43.68	328.06 ± 40.56

values are presented as means ± standard deviation (SD; $n = 3$). The significance in difference between CG and EG groups was tested using Student's *t*-test at the level at $P < 0.05$. Data within the same column without any superscript are not different

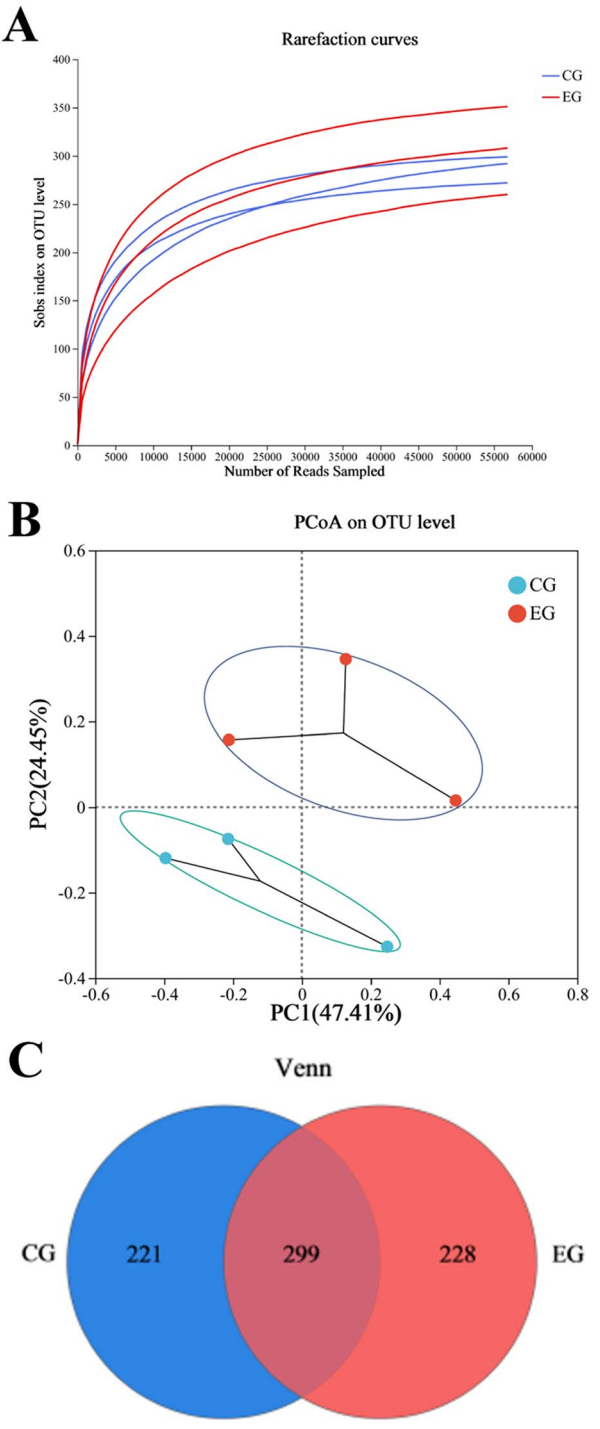

Fig. 4 Rarefaction curves (**A**), PCoA analysis (**B**), and Venn diagram (**C**) of intestinal microbiota in *P. clarkii* between CG and EG groups at OUT level. CG, control group; EG, 0.60% inulin-added group

communities were not significantly different between the two groups ($P > 0.05$). Moreover, partial least squares discriminant analysis demonstrated that samples in CG or EG groups were clustered in different regions of the PCoA plot, respectively, manifesting a clear separation of the intestinal bacteria between CG and EG groups (Fig. 4B). The Venn diagram revealed that the CG and EG groups possessed 221 and 228 OTUs alone, respectively, and that the two groups shared 299 OTUs (Fig. 4C).

Changes in the composition and predicted functions of intestinal microbiota

The relative abundance maps of intestinal microbiota in CG and EG groups are demonstrated in Fig. 5. Five dominant phyla were found among the two groups, with a significant increase in the relative abundance of Proteobacteria in the EG group relative to the CG group, and a corresponding decrease in Firmicutes, Bacteroidetes, Actinobacteria, and Patescibacteria in the EG group (Fig. 5A). At the genus level, the relative abundance of *Citrobacter* and *Acinetobacter* spp. in the EG group was significantly higher relative to the control group (Fig. 5B).

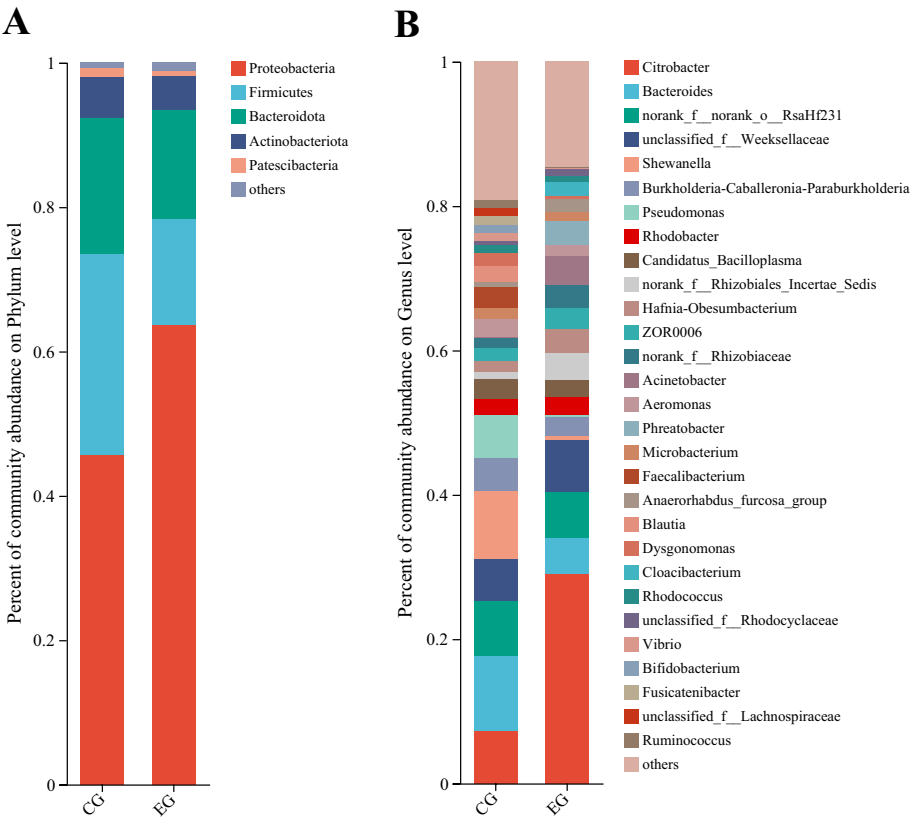

Fig. 5 Relative abundance of intestinal microbial community of *P. clarkii* at the phylum level (**A**) and at the genus level (**B**) between CG and EG groups. CG, control group; EG, 0.60% inulin-added group. The bar graphs represented microbiota composition

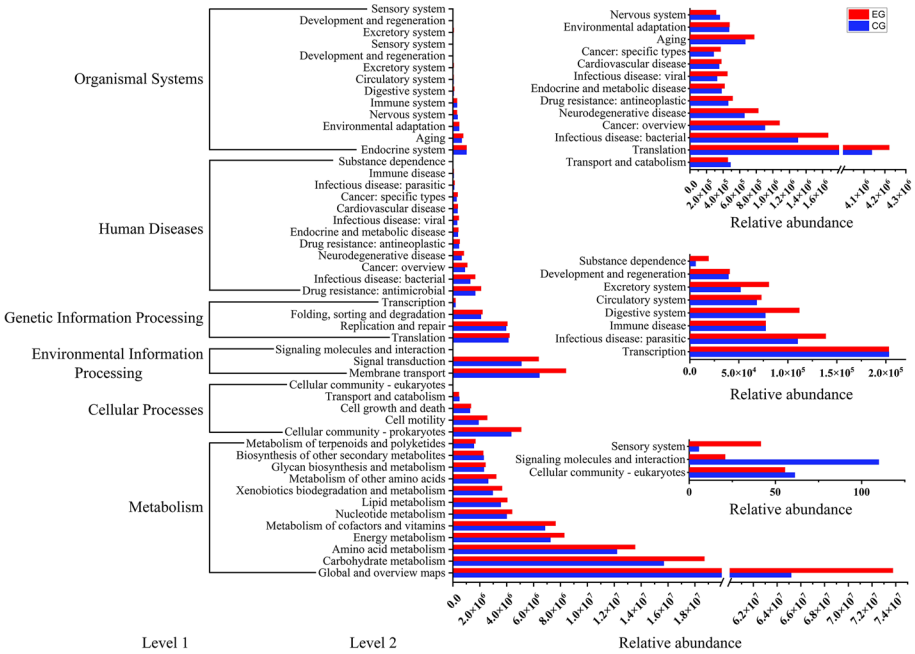

Fig. 6 KEGG functional classification of intestinal bacteria in *P. clarkii*. CG, control group; EG, 0.60% inulin-added group. The *x*-axis represented KEGG categories; the *y*-axis represented the relative abundance of metabolic pathways

The predicted bacterial gene functions using PICRUSt are demonstrated in Fig. 6. The result indicated that, first, diets supplemented with inulin upregulated several metabolism-related pathways in the EG group, such as carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, lipid metabolism, xenobiotics biodegradation and metabolism, and metabolism of other amino acids. Secondly, dietary inulin supplementation upregulated pathway related to organismal systems, such as digestive system, circulatory system, and excretory system. Moreover, dietary inulin positively regulates pathways associated with cellular processes, such as cellular community-prokaryotes and cell motility, and environmental information processing-related pathway including membrane transport and signal transduction.

Discussion

Dietary inulin enhanced the growth performance of *P. clarkii*

Dietary supplemented with 0.6% inulin showed a significant increase in SR, WG, and SGR and a significant decrease in FCR, indicating that appropriate dietary inulin supplementation can improve the growth performance of crayfish. HIS is considered an indicator of body health and energy reserves (Khan et al. 2015), and in this study, HIS increased significantly with the increase of inulin supplementation (Kong et al. 2021), indicating better nutritional status or higher food assimilation rate and growth

rate (Sureshkumar and Kurup 1999). The improvement in *P. clarkii* growth performance following dietary supplementation with inulin may be attributed to a number of factors, such as enhanced antioxidant defenses, improved intestinal health, and activities of digestive enzyme (Fu et al. 2022).

Dietary inulin changed the body composition and improved the flesh quality of *P. clarkii*

Muscle is the mostly main edible part of crayfish food product and its CP and CL contents are indicators of the quality and nutritional level of crayfish food (Ali et al. 2016). In this study, dietary supplementation with 0.4% prebiotic inulin significantly increased the CP, CL, and ash contents of muscle, suggesting that the supplemented prebiotic inulin was effective in improving the quality of abdomen meat under this rearing conditions. Higher doses of inulin showed a similar trend of altering CP and CL content throughout the whole body. Previous studies on crustaceans have reported the prebiotic impact on the proximate composition (Mazlum et al. 2011; Sang et al. 2011, 2014), while the study regarding effects of inulin on the proximate composition of muscle and whole body was extremely limited. The combined addition of mannan oligosaccharide (MOS) and inulin did not significantly alter the proximate composition of whole body in *Eriocheir sinensis* (Lu et al. 2019); however, the body ash content in *L. vannamei* tended to increase with the increasing level of inulin (Zhou et al. 2020). Indeed, the alternation of biochemical components due to prebiotics, including inulin, frequently shows diversity and inconsistency in different cultured aquatic animals (Ali et al. 2016; Daniels and Hoseinifar 2014), which may be attributed to some vital factors such as life stage, species, feeding, and rearing condition (Ghafarifarsani et al. 2021).

Dietary inulin enhanced the antioxidant capacity and immunity of *P. clarkii*

The antioxidant defense system is critical for crustaceans to resist external stress, especially their lack of adaptive immunity, and this system is vulnerable to diet, external environment, and health status (Frías-Espericueta et al. 2022; Hoseinifar et al. 2020). The hepatopancreas of crustacean is very sensitive to oxidant status (Zheng et al. 2022). In the present study, MDA content and antioxidant indices (T-AOC, T-SOD, CAT, and GSH-PX) in hepatopancreas decreased and increased significantly after the addition of 0.4–0.6% dietary inulin, respectively. A study showed that 0.2–0.4% dietary inulin significantly increased SOD and CAT activities and reduced MDA content of *L. vannamei* (Zhou et al. 2020). The mechanisms by which inulin enhances antioxidant defenses are complex. Firstly, inulin can act as an ROS scavenger itself (Shang et al. 2018; Stoyanova et al. 2011); secondly, inulin may indirectly scavenge ROS through the production of antioxidant enzymes and short-chain fatty acids (SCFA) (Pourghassem Gargari et al. 2013); moreover, inulin may improve the antioxidant activity of the host by modulating the composition of the intestinal bacteria (Pasqualetti et al. 2014).

Besides enhancing the antioxidant defense system, we also confirmed that dietary inulin improved the immune performance of *P. clarkii*. In the present study, we found that dietary inulin significantly increased the relative expression of immune-related genes *Lectin*, *ALF*, and *Crustin*. Our recent study has indicated that the activities of acid phosphatase, alkaline phosphatase, and lysozyme were significantly improved in

hepatopancreas of *P. clarkii* fed with 0.60% dietary inulin (Fu et al. 2022). Actually, inulin can act as an immunosaccharide binding to pattern recognition receptors (PRRs) on innate immune cells to stimulate innate immunity (Song et al. 2014; Vogt et al. 2015). In addition, intestinal fermentation products of inulin, such as fructooligosaccharides (FOS), can interact with TLR2, a membrane surface receptor expressed on macrophages, thus promoting innate immunity and expression level of *crustin1*, *lysozyme*, and *SOD* in *P. clarkii* (Dong and Wang 2013). In conclusion, inulin can be used as an immunostimulant to enhance the innate immunity of *P. clarkii*.

Dietary inulin modulated the intestinal microbiota and improved the intestinal health status

In this study, crayfish supplemented with 0.6% inulin in their diets showed the best growth performance, as well as better antioxidant and immune performance, so crayfish in the 0.6% dietary inulin group were selected for intestinal microbial testing and analysis. Diets supplemented with 0.6% inulin significantly altered the diversity and abundance of intestinal microbiota of *P. clarkii*, with a significant increase in the relative abundance of *Citrobacter* spp. Inulin is a readily fermentable fiber by intestinal bacteria, generating large quantities of short-chain fatty acids (SCFA) (Asadpoor et al. 2021), and we hypothesized that some SCFA may be converted to citric acid by biochemical reactions in the crayfish gut, resulting in a significant increase in the relative abundance of *Citrobacter* spp. The bacteria of genus *Citrobacter* have metabolic potential to produce the chitin/chitosan, which are constituted by the monomers of N-acetylglucosamine (GlcNAc) and/or glucosamine (GlcN) (Takeo et al. 2018), and the growth and development of crustaceans are majorly associated with the biosynthesis of chitin, the pathways of which can be started from GlcNAc and/or GlcN (Zhang et al. 2021). Moreover, inulin addition resulted in a significant increase in the relative abundance of the genus *Acinetobacter*. A previous study demonstrated that *Acinetobacter* strains grow well on SCFA but not on simple carbohydrates, suggesting that SCFA can promote the proliferation of *Acinetobacter* (Kim et al. 1997). *Acinetobacter* spp. have the ability to metabolize amino acids, aromatic compounds, and short-chain fatty acids, as well as contribute to carbohydrate metabolism in animals (Dworkin et al. 2006), and may prevent the activation of virulence factors and contribute to resistance to infection by other pathogenic bacteria (Alfiansah et al. 2020).

The overall effect of exogenous substances on the gut health of animals can be identified by the PICRUSt KEGG functional analysis. For instance, the addition of 0.4% inulin to the diet significantly altered the metabolism, genetic information processing, cellular processes, and organismal system-related pathways in *L. vannamei* (Zhou et al. 2020). In the present study, the results of PICRUSt analysis showed that the intestinal microbiota of the inulin-added group significantly promoted the processes of metabolism, digestion, transport, circulation, and cellular processes in *P. clarkii*, indicating the improvement of the intestinal health of *P. clarkii* by the dietary addition of inulin.

Conclusions

In summary, this study revealed that dietary inulin significantly improved the growth performance and the flesh quality, as well as the antioxidant capacity and the relative expression of immune-related genes in *P. clarkii*. A broken-line regression analyses of FBW and SGR indicated that the optimal dietary inulin level was approximately 0.7%. In addition, 0.6% dietary inulin was beneficial to intestinal health by modulating the intestinal microbiota, which might be associated with the altered nutrient metabolism and the improved host growth performance. Thus, prebiotic inulin had a beneficial effect on improving the growth, antioxidant capacity, non-specific immunity, and intestinal microecology of crayfish. These results will provide a theoretical reference for dietary preparation and healthy culture of *P. clarkii*.

Author contribution Qian Wang and Guoliang Ruan: conceptualization, methodology, and writing—review and editing. Yanbin Lin, Wenhao Fan, and Shengxuan Li: investigation, data curation, and writing—original draft. Heng Zhang: methodology, investigation, and writing—review and editing. Liu Fang: software, validation, visualization, and project administration. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author, Guoliang Ruan, upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Qian Wang[1,3] · Yanbin Lin[1,3] · Heng Zhang[2] · Wenhao Fan[1,3] · Shengxuan Li[1,3] · Guoliang Ruan[1,2,3] · Liu Fang[1,2,3]

✉ Guoliang Ruan
ruanguoliang@126.com

✉ Liu Fang
fangliu@yangtzeu.edu.cn

[1] The Innovative Technology Research Center of Crayfish Breeding and Healthy Farming, Yangtze University, Jingzhou 434025, People's Republic of China

[2] Hubei Fisheries Industrial Technology Research Institute, Jingzhou 434026, People's Republic of China

[3] The Innovative Center of Animal Nutrition and Feed Application Technology, Yangtze University, Jingzhou 434024, People's Republic of China