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Short-term dietary supplementation of β-glucan and chitosan modulates the immune response in freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879)

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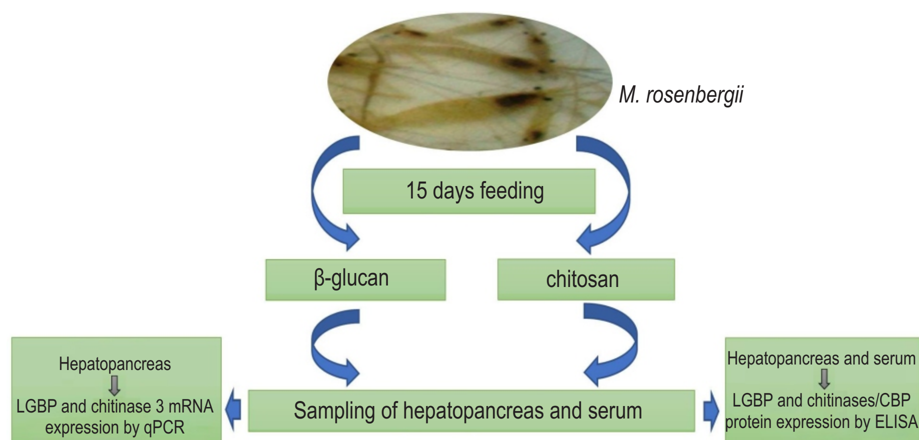
Abstract

Aim: The present study evaluates the immunomodulatory potentials of β-glucan and chitosan administered orally for 15 days to the freshwater prawn, *Macrobrachium rosenbergii* juveniles.

Methodology: Two diets were formulated supplementing 1.5 g kg^{-1} and 20 g kg^{-1} of β-glucan (diet G) and chitosan (diet C), respectively, with a control diet (diet B). The prawns were fed twice daily at 5% biomass. At the end of the feeding trials, mRNA expression profiles of immune-related genes lipopolysaccharide- and β-1,3-glucan binding protein (LGBP) and chitinase 3 were analyzed in qPCR. Corresponding LGBP protein and chitinases/CBP (chitinase 1, chitinase 1C and obstructor E, a chitin-binding protein) protein levels were also estimated by ELISA.

Results: Supplementation of the immunostimulants (diets G and C) significantly enhanced the mRNA expression of both LGBP and chitinase 3 in the hepatopancreas. The protein expression of LGBP and chitinases/CBP was also significantly increased in the serum and hepatopancreas samples of both treatment groups.



Interpretation: The short-term supplementation of β-glucan and chitosan in diets demonstrated beneficial effects in *M. rosenbergii* by enhancing the expression of important immune molecules like LGBP and chitinases, indicating their potential applications during the period of biotic stress.

Key words: β-glucan, Chitinase, Hepatopancreas, Immunostimulants, LGBP, *Macrobrachium rosenbergii*

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Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (Subphylum- Crustacea; Class- Malacostraca; Order- Decapoda; Family- Palaemonidae) is indigenous to South Asian countries like India, Bangladesh, Myanmar, Thailand and Malaysia (Tan and Wang, 2022), and is commercially exploited with an annual revenue of 2.56 billion USD in 2022 (FAO, 2024). However, the semi-intensive aquaculture practices have led to infectious disease outbreaks of bacterial and viral origin affecting the culture of the species and leading to economic losses. Interventions such as administration of antibiotics, probiotics, synbiotics, immunostimulants, and vaccine are in practice for combating infections and improving the defense status of the host species against microorganisms (Mohan *et al.,* 2019; Kumar *et al.,* 2023). To be particular, immunostimulants are substances that can stimulate the immune system of the host and thereby, increase its ability to fight against infectious diseases (Wang *et al.,* 2008). Besides, these are considered to be safe and cost-effective and as per the current trend are derived from natural sources. Based on the source, immunostimulants are classified into several groups *viz.,* bacterial-, algal-, animal-derived, nutritional factors and hormones or cytokines (Kumar *et al.,* 2023).

Among the various immunostimulants tried in aquaculture, polysaccharides like β-glucan and chitosan are the most popular as these are easily available, affordable and sustainably derived. While β-glucan is a polymer of D-glucose linked together either by β-1,3- or β-1,6-glycosidic bonds, chitosan (deacetylated product of chitin) is a linear copolymer of N-acetyl-D-glucosamine and D-glucosamine linked by β-1,4-glycosidic bonds. β-glucan forms the structural component of the cell walls of yeast, algae, fungi and bacteria and chitosan has been primarily derived from insect and crustacean shells (Wang *et al.,* 2008; Yang *et al.,* 2014; Kim *et al.,* 2017). Apart from their applications in food, cosmetics, and pharmaceutical industries (Chen and Chen, 2019), they have been tested as dietary supplements enhancing the immunostimulatory potential of crustaceans against microbial pathogens, besides growth enhancement potential (Goh *et al.,* 2022).

However, Bai *et al.* (2010) have cautioned that continuous feeding of immunostimulants for long periods can cause immune fatigue eliminating its beneficial effects. β-glucan supplemented feed has been shown to enhance the survival of juvenile *Litopenaeus vannamei* infected with *Vibrio parahaemolyticus* (Felix *et al.,* 2022), *Penaeus monodon* against white spot syndrome virus (WSSV) (Chang *et al.,* 2003), *Fenneropenaeus chinensis* against *V. parahaemolyticus* (Wang and Qi, 2010), and *M. nipponense* against *V. parahaemolyticus* (Tian *et al.,* 2023). Chitin and chitosan too either through systemic injection or in the diet have been shown to increase the immune ability and resistance to *V. alginolyticus* infection in shrimp, *L. vannamei* (Wang and Chen, 2005; Cheng *et al.,* 2021). In *M. rosenbergii*, the dietary supplementation of β-glucan (long-term, for a period of 60 days) led to increased survival of the juveniles to *Aeromonas hydrophila* infection (Meshram *et al.,* 2015). Similarly, chitin-diet fed *M. rosenbergii* showed enhanced resistance to white-tail disease caused by *M. rosenbergii* nodavirus (*Mr*NV) (Naveen *et al.,* 2015). These isolated reports, however, did not discuss the influence of immunostimulatory diets on genes that may act as pattern recognition receptors (PRRs) and influence the innate immune cell signaling cascades in prawns or shrimps. PRRs recognize invading pathogens as non-self by binding to pathogen associated molecular patterns (PAMPs) present on the membrane of pathogens eliciting humoral and cell-mediated immune responses (Patnaik *et al.,* 2024).

Lipopolysaccharide- and β-1,3-glucan binding protein (LGBP) is a multifunctional PRR known to elicit the activation of prophenoloxidase (proPO) cascade among others and even possess lectin-like properties (Du *et al.*, 2019; Sahoo *et al.,* 2023). Further, Sahoo *et al.* (2023) have developed an enzyme-linked immunosorbent assay (ELISA) for the quantitation of LGBP protein in *M. rosenbergii* and showed its increased induction during bacterial challenge. LGBP is also known to be modulated in crustaceans with dietary supplementation of immunostimulants such as β-glucan (Luan *et al.,* 2021; Shen *et al.,* 2023), alginate and spirulina (Azhar and Yudiati, 2023). Crustacean chitinases have been suggested to be involved in innate immunity, apart from the molting and growth process, and digestion of chitin-rich food (Zhang *et al.,* 2021). For instance, chitinases participate in the toll signaling pathway of *Procambarus clarkii* (Liu *et al.,* 2021). Recently, Sahoo *et al.* (2024) reported enhanced induction of chitinases/CBP (consisting of chitinase 1, chitinase 1C and obstructor E, a chitin-binding protein (CBP) in *M. rosenbergii* in response to *V. harveyi* challenge indicating its possible involvement in the immune response. In the present study, it is hypothesized that short-term supplementation of β-glucan and chitosan can modulate the expression of immune genes such as LGBP and chitinases, thus improving the defense status of the freshwater prawn, *M. rosenbergii.*

Materials and Methods

Experimental diet preparation: The basal diet (diet B) consisted of groundnut oil cake (40%), soybean meal (20%), fish meal (20%), rice bran (17%), carboxymethyl cellulose (0.1%) and vitamin-mineral mix (3%). The vitamin-mineral mix was a commercial product namely PREMIX PLUS from PVS Laboratory Limited, India that contained vitamin A, 5,500,000 IU; vitamin D3, 1,100,000 IU; vitamin B2, 2000 mg; vitamin E, 750 mg; vitamin K, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mcg; calcium pantothenate, 2,500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2000 mg; Co, 450 mg; L-lysine, 10 g; DL-methionine, 10 g; selenium, 50 ppm; Satwari, 2,500 mg per 2.5 kg of mix. The basal diet was supplemented with immunostimulants to formulate two experimental diets, 'diet G' containing β-glucan (MacroGard, Biotech Pharmacon, ASA, Norway) and 'diet C' containing deacetylated chitosan (Sigma, USA). The dose of β-glucan was selected as 1.5 g kg^{-1} based on our earlier experiment conducted

on *M. rosenbergii* (Sahoo *et al.,* 2008). Since, the dose of chitosan was not available for *M. rosenbergii,* the dose @ 20 g kg^{-1} reported for shrimp, *P. monodon* juveniles (Rochana *et al.,* 2019) was selected for use. The immunostimulants were incorporated into diet B by adjusting the overall amount of rice bran in the feed. The ingredients of each diet were weighed, mixed, sieved and made into dough with water. This was passed through an extruder and dried at 30 °C. After drying, the feed was passed through a pelletizer and 2 mm size sinking pellets were prepared. The pellets were kept in air-tight containers until further use.

Prawn and feed trial design: One hundred twenty juvenile prawns (3.1-5.8g size) were sourced from the prawn hatchery unit, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India. The prawns were acclimatized in the wet laboratory in 500 l tanks under continuous aeration and fed *ad libitum* with a commercial pellet feed. During the experiment, the water quality parameters were: temperature - 28-29 °C, pH - 8.0-8.5, alkalinity - 90-103 mg l^{-1}, total hardness - 85-100 mg l^{-1}, ammonia - 0.008-0.012 mg l^{-1} and dissolved oxygen - 6.0-6.5 mg l^{-1}. The trials were conducted in two different sets, one set consisted of diet B and diet G, while the other was diet B and diet C.The initial weight of each prawn was taken and distributed randomly into each tank. The experiment was carried out in triplicate with each tank consisting of 10 prawns, and 30 prawns in total in each diet group. The prawns were fed twice daily @ 5% of their body weight with the respective diets for 15 days. Approximately, 50% of water was exchanged daily with the removal of uneaten feed and fecal matter.

Hemolymph and hepatopancreas tissue sampling: At the end of 15 day trial, the prawns were kept without feed for 24 hr and the hemolymph was collected through pericardial sinus and allowed to clot at room temperature for separating serum. Subsequently, the prawns were anesthetized with 0.01% clove oil and hepatopancreas samples were collected and weighed. Individual samples were homogenized by a bead homogenizer (Super FastPrep-1 homogenizer, MP Biomedicals, USA) in 30% (w/v) TBS-$CaCl_2$ (20 mM tris base, 0.15 M NaCl, 3 mM $CaCl_2$, pH 7.4) containing protease inhibitor (Halt Protease Inhibitor Cocktail 100X, Thermo Scientific, USA). The homogenate was centrifuged at 9500 xg for 20 min and the supernatant collected was stored at -20 °C until further use. During sampling, approximately 100 mg of hepatopancreatic tissue from 3 random individual prawns from each tank were collected in RNAlater (Sigma, USA) and kept at -80 °C for gene expression studies.

Gene expression analyses of LGBP and chitinase 3 in hepatopancreas: mRNA expression analysis of immune-related genes *viz.*, LGBP and chitinase 3 were studied in the hepatopancreas of prawns fed with immunostimulant supplemented diets G and C, against respective diet B. Total RNA was extracted from the hepatopancreas using TRI reagent (Sigma, USA) following the protocol of Simms *et al.* (1993). The isolated RNA was treated with DNase I (Genei, India) at 37 °C for 1 hr after which the DNase I was inactivated with 50 mM EDTA at 65 °C for 10 min. Subsequently, RNA was quantitated using BioSpectrometer (Eppendorf, Germany) and cDNA was synthesized from 1 µg of DNase-treated RNA by RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA), following the manufacturer's instructions. Briefly, 1 µl of Oligo $(dT)_{18}$ primer was added to 1 µl of RNA (1 µg) and nuclease-free water, mixed and incubated at 65 °C for 5 min. The reaction mixture was cooled on ice, followed adding 5X reaction buffer (4 µl), RNase inhibitor (1 µl), 10 mM dNTP (2 µl) and reverse transcriptase (1 µl). The reaction was carried out at 42 °C for 60 min and was terminated at 70 °C for 5 min.

Primers for chitinase 3 gene (Forward: CTT GGC TGG TTG TAT GGT and Reverse: GGT TGG TGG TCT TGT GAA) were designed using the Primer Premier 5 (version 5.0, Premier Biosoft International, Palo Alto, CA) software using the available nucleotide sequences from the NCBI database. Primers for LGBP (Forward: ACC AAT ACG GAG GAA CCA and Reverse: CCA GAT GTT GTC AAT GTT GT) and β actin (Forward: CAT CAC CAA CTG GGA CGA CAT GGA and Reverse: GAG CAA CAC GGA GTT CGT TGT) were used as per Sahoo *et al.* (2023). The specificity of the primers was checked through amplification and gel electrophoresis of cDNA. The reaction mixture comprised of cDNA (1 µl), 10X PCR buffer (2.5 µl), 10 mM dNTP (0.5 µl), forward and reverse primer (0.5 µl each) and Taq polymerase (0.25 µl). The volume of the reaction mixture was made up to 25 µl with nuclease free water. PCR amplification was carried out at 95 °C for 5 min, followed by 34 cycles at 94 °C for 30 sec, 54 °C for 30 sec for LGBP gene/57 °C for 30 sec for chitinase 3 gene and β actin at both the temperatures, 72 °C for 45 sec and final extension at 72 °C for 7 min. The amplified products were electrophoresed at 2% agarose concentration containing ethidium bromide. Further, the mRNA expression of LGBP and chitinase 3 were analyzed using Light Cycler 96 SW 1.1 (Roche, Germany) with the reaction mixture comprising of 2X KAPA SYBR FAST master mix (Kapa Biosystems, South Africa), forward and reverse primers, cDNA and PCR grade water, as per Sahoo *et al.* (2023). PCR reaction cycles were also carried out following Sahoo *et al.* (2023), setting annealing temperature at 54 °C and 57 °C for LGBP and chitinase 3, respectively.

The relative mRNA expression levels were evaluated using the comparative Cq method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001). The expression levels were normalized to *M. rosenbergii* β-actin serving as endogenous control.

Quantitation of LGBP and chitinases/CBP in hepatopancreas and serum samples: LGBP and chitinases/CBP concentrations in hepatopancreas and serum samples from both diet groups were quantitated by previously developed ELISAs; a sandwich ELISA for LGBP (Sahoo *et al.,* 2023) and an indirect ELISA for chitinases/CBP (Sahoo *et al.,* 2024). Rabbit polyclonal antisera against these proteins were available in the laboratory and used in these experiments. For LGBP quantitation, hepatopancreas and serum samples were used at respective dilutions of 1:16000 and 1:12.5. For chitinases/CBP quantitation, the samples were used at 1:32000 and 1:250 dilutions,

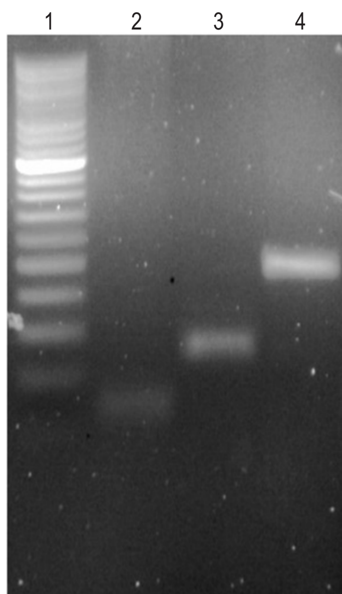

Fig. 1: Agarose gel electrophoresis of PCR amplified products; Lane 1: 50 bp ladder, Lane 2: β actin gene, Lane 3: chitinase 3 gene, Lane 4: LGBP gene.

respectively. The concentrations determined were expressed in mg g^{-1} tissue for hepatopancreas and µg ml^{-1} for serum.

Statistical analysis: The results obtained from the qPCR for LGBP and chitinase 3 gene expression in hepatopancreas were represented as mean±SE of three biological replications and, the statistical significance between the treatment and respective control groups was calculated by Student's t-test ($p<0.05$). Similarly, the concentrations of LGBP and chitinases/CBP proteins in hepatopancreas and serum samples determined in ELISAs were also represented as mean±SE with the significance tested by Student's t-test.

Results and Discussion

Crustaceans over-rely on innate immune mechanisms for confronting pathogens as they lack adaptive immune system as in vertebrates. Hemocytes help in cellular defense by eliciting an attack on the pathogens via phagocytosis, melanization, encapsulation, and cell lysis (Patnaik *et al.,* 2024). On the other hand, the PRRs recognize PAMPs on the pathogen surface and mount signaling cascades leading to the transcription of effector molecules (antimicrobial peptides) (Patnaik *et al.,* 2024). In our previous studies, we have characterized a few PRRs in *M. rosenbergii* that showed increased expression after bacterial and viral challenge and were putatively involved in innate immunity (Patnaik *et al.,* 2021; Baliarsingh *et al.,* 2022; Sahoo *et al.,* 2023; Sahoo *et al.,* 2024). Specifically, LGBP showed an increased induction in *M. rosenbergii* serum and hepatopancreas under challenged conditions (Sahoo *et al.,* 2023). In another study, chitinases were induced in the hepatopancreas of *V. harveyi*-challenged *M. rosenbergii* (Sahoo *et al.,* 2024). In this work, we have proved that short-term feeding of immunostimulants such as β-glucan and chitosan can induce the expression of LGBP and chitinase in juvenile prawns, thus improving the defense status. Juvenile stages of prawns are more prone to pathogen attacks as most of their energy resources are focused on the growth and development (Hauton *et al.,* 2007). Further, the dosage and duration of immunostimulants use are critical parameters for preventing low disease resistance and immune system suppression (Licona-Jain *et al.,* 2022).

An overdose or longer period of application of any immunostimulant may lead to an immune-compromised state rendering the animal to succumb to infections (Bai *et al.,* 2010). In this study, a feeding trial of 15 days was conducted to boost the immune response. Such short-term feeding experiments with β-glucan have been conducted in a number of shrimp species with the depiction of immune enhancement. A 7 and 14 days feeding of β-glucan to adult *M. rosenbergii* enhanced their immune status (Sahoo *et al.,* 2008). Even, oral administration of 0.2% glucan for only 3 days to adult *P. monodon* significantly increased the hemolymph PO-activity (Suphantharika *et al.,* 2003). Also, *L. vannamei* juveniles fed with a β-glucan diet every alternate day for 15 days showed higher survival rate against *V. parahaemolyticus* challenge (Felix *et al.,* 2022). Short-term chitosan feeding similar to our experiment (2 week) have demonstrated enhanced immunity and health status in some fish species such as Nile tilapia, *Oreochromis niloticus* (Fadl *et al.,* 2020) and golden pompano, *Trachinotus ovatus* (Yu *et al.,* 2023).

The cDNA amplification products with the designed primers showed specific single bands in the gel with expected product sizes of 68, 130 and 242 bp respectively for β actin, chitinase 3 and LGBP genes (Fig. 1) and used in subsequent qPCR analyses. The effects of diets G and C fed for 15 days to *M. rosenbergii* juveniles showed significant upregulation of LGBP and chitinase 3 mRNA expression in the hepatopancreatic tissue in comparison to the control group (Fig. 2 a-d). LGBP is one such PRR that recognizes and binds to LPS and β-glucan present on the surface of pathogens which subsequently triggers innate immunity. In crustaceans, LGBP is mostly expressed in the hepatopancreas and upon binding to pathogens induce various cellular responses such as phagocytosis, encapsulation and nodule formation (Wang *et al.,* 2007). Similar upregulation of LGBP gene has also been reported in the hepatopancreas of *L. vannamei* fed with a β-glucan supplemented diet (Wang *et al.,* 2008). Further in *L. vannamei,* Wongsasak *et al.* (2015) observed an increase in LGBP transcripts in the hemolymph when fed with β-glucan supplemented diet for 90 days and Shen *et al.* (2023) also showed enhanced LGBP transcripts in the intestine after feeding with β-glucan supplemented diet for 60 days. Cheng *et al.* (2021), however, observed that *L. vannamei* diet supplemented with chitosan did not affect the mRNA expression of LGBP in both hepatopancreas and hemocytes samples. This is in contrast to our findings where the mRNA expression of LGBP in the

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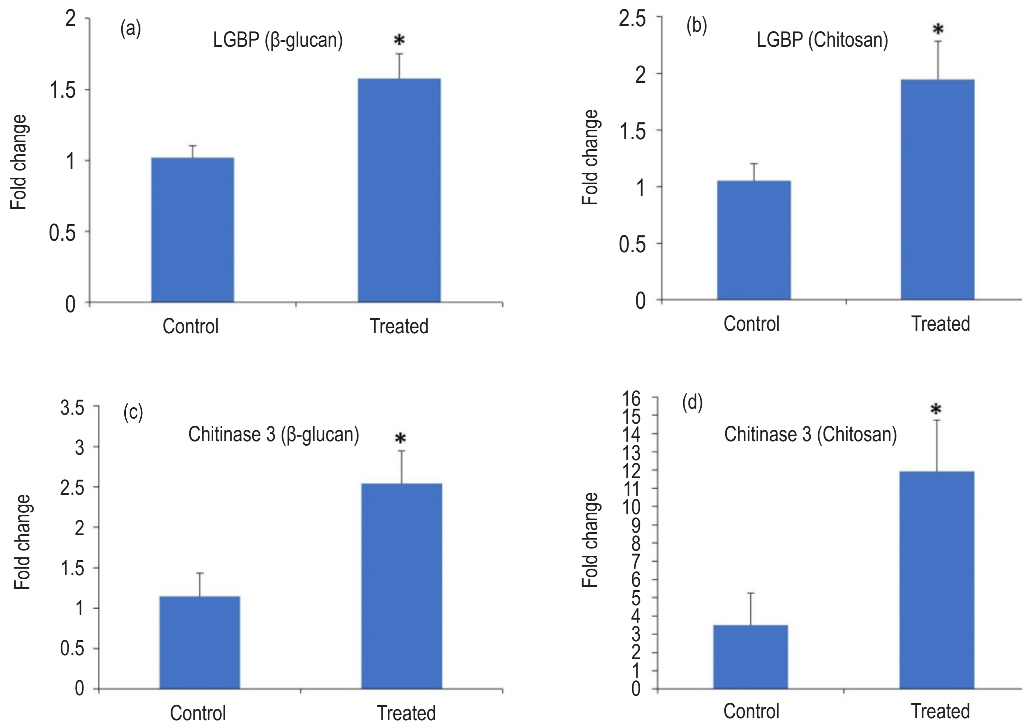

Fig. 2: qPCR analysis of LGBP gene in the hepatopancreas of *M. rosenbergii* fed diets supplemented with β-glucan (a) and chitosan (b) and, chitinase 3 gene in hepatopancreas of *M. rosenbergii* fed diets supplemented with β-glucan (c) and chitosan (d). *indicates statistically significant from the respective control group at $p<0.05$.

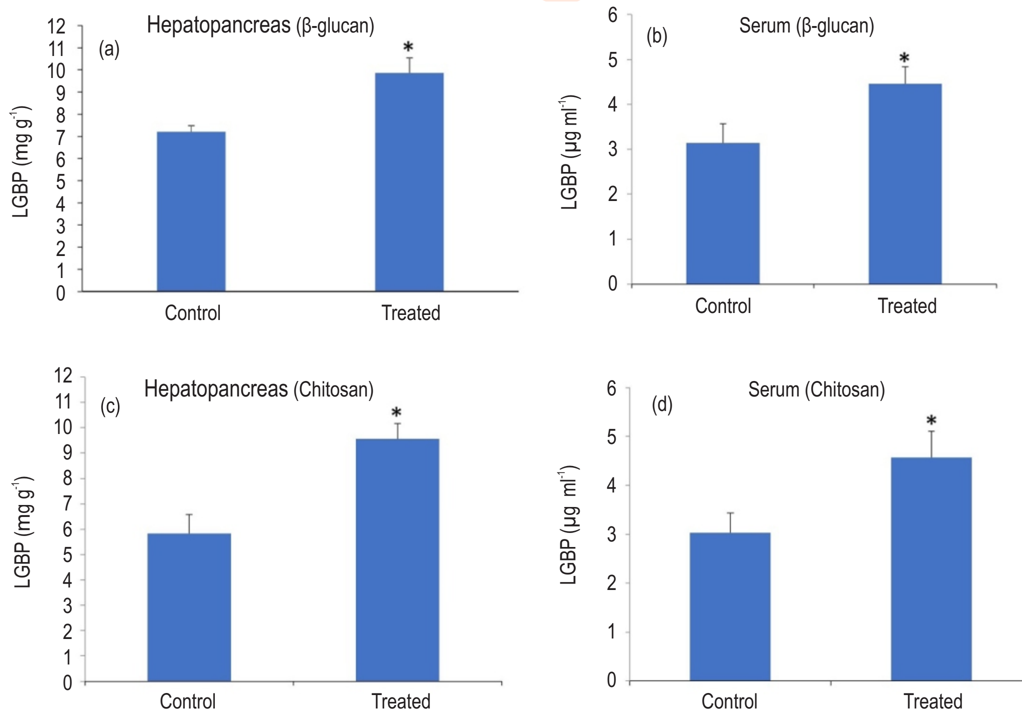

Fig. 3: Quantitation of LGBP in the hepatopancreas (a) and serum (b) of diet group G and in the hepatopancreas (c) and serum (d) of diet group C. *indicates statistically significant from the respective control group at $p<0.05$.

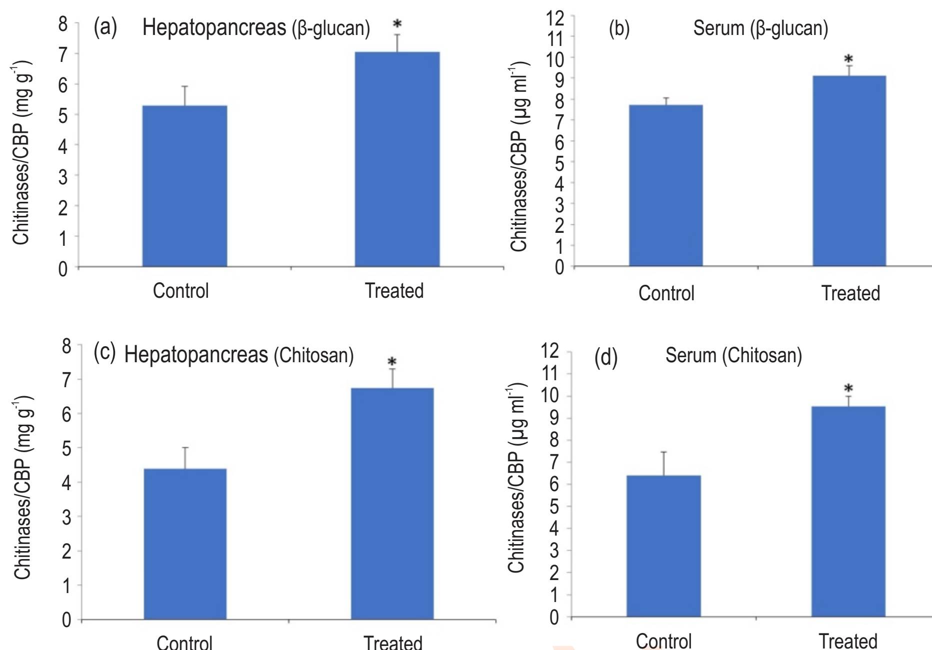

Fig. 4: Quantitation of chitinases/CBP in the hepatopancreas (a) and serum (b) of diet group G and in the hepatopancreas (c) and serum (d) of diet group C. *indicates statistically significant from the respective control group at $p<0.05$.

hepatopancreas was significantly upregulated after short-term chitosan supplementation in the diet.

Chitinase 3 was selected in the present investigation for mRNA expression study since this was the only chitinase in *M. rosenbergii* whose nucleotide sequence is available in the NCBI database. Besides, the hepatopancreas was selected as the preferred tissue for this analysis, as chitinases have been reported to be primarily expressed in the hepatopancreas of many prawn species (Zhang *et al.,* 2021). Chitinase 3 has been reported to be associated primarily with the degradation of chitin-containing food (Zarantoniello *et al.,* 2023) and hence, its higher expression in the chitosan-supplemented diet has been shown in *L. vannamei* (Tseng *et al.,* 2021) similar to our study. Even in β-glucan supplemented feed, the expression of chitinase gene was found to be enhanced, which was quite unexpected. There is no previous report showing chitinase expression in glucan-supplemented diet and this needs further study to explain. However, chitinase in *M. rosenbergii* also possess lectin-like properties of binding to carbohydrates (Sahoo *et al.,* 2024) and that might be the reason for enhanced chitinase activity with β-glucan supplementation.

The results of sandwich ELISA for LGBP protein concentrations in hepatopancreas and serum samples showed a significant increase in both G and C diet groups compared to respective diet B (Fig. 3 a-d). The average concentration of LGBP in the hepatopancreas of the group fed with β-glucan was 9.86±0.68 mg g^{-1} tissue, while that of the control group was 7.23±0.27 mg g^{-1} tissue. The average concentration in the serum samples was 4.46±0.37 µg ml^{-1} in the β-glucan group against the control group having 3.14±0.42 µg ml^{-1}. Similarly, the group fed with chitosan supplemented diet showed a significantly higher LGBP concentration of 9.56±0.6 mg g^{-1} tissue in hepatopancreas and 4.57±0.53 µg ml^{-1} in serum samples against the control group showing a concentration of 5.82±0.75 mg g^{-1} and 3.03±0.4 µg ml^{-1}, respectively. The significant increase in the expression of LGBP proteins shown in the hepatopancreas and serum of both the experimental diet groups G and C strongly support our earlier RT-qPCR findings of an elevated expression of LGBP mRNA. This is possibly the first report of chitosan feeding in crustaceans that showed enhanced expression of LGBP both at mRNA and protein levels. Sahoo *et al.* (2023) reported that LGBP in *M. rosenbergii* possess lectin-like activity of binding to carbohydrates and this possibly could be the reason for chitosan modulating the enhanced activity of LGBP. However, further study is needed to explain how chitosan induces this LGBP expression. Overall, the increase in the expressions of LGBP mRNA and protein indicates the immunomodulatory effects of both supplements.

The expression of chitinases/CBP proteins showed a significant increase in the hepatopancreas as well as serum fed with the experimental diets in comparison to control diet (Fig. 4 a-d). The concentration of chitinases/CBP in the hepatopancreas and serum of diet group G were 7.05±0.56 mg g^{-1} tissue and 9.12±0.47 µg ml^{-1}, respectively in comparison to the control group with a concentration of 5.29±0.62 mg g^{-1} tissue in the hepatopancreas and 7.72±0.33 µg ml^{-1} in the serum. The concentration in the hepatopancreas of diet group C was

6.74±0.55 mg g^{-1} tissue and in serum was 9.54±0.43 µg ml^{-1}. The average concentration of the respective control group was 4.39±0.61 mg g^{-1} tissue in the hepatopancreas and 6.4±1.06 µg ml^{-1} in the serum samples. The enhanced expression of chitinases/ CBP against *V. harveyi* bacterial challenge has been shown earlier (Sahoo *et al.,* 2024). In the present study, the significant increase in the protein expression of chitinases/CBP in the hepatopancreas and serum samples of both the supplemented diet groups indirectly supports the enhanced expression of chitinase 3 gene as reported in our previous RT-qPCR studies. Thus, it confirms the modulation of a number of chitinases by the administered immunostimulants and their possible participation in the digestion of supplemented food and immune responses of prawns. Similar immune stimulation by LPS and peptidoglycan has shown upregulation of chitinase expression in the hepatopancreas of *P. clarkii* (Liu *et al.,* 2021).

Short-term supplementation of β-glucan and chitosan in diet leads to an enhanced immune surveillance in the freshwater prawn, *M. rosenbergii,* exemplified by increased expression of immune-related genes such as LGBP and chitinase 3. This was supported by increased induction observed at the protein level. Although not studied, the immunostimulatory potential of β-glucan and chitosan could make the prawns more resistant to invading pathogens. However, our observations on the expression of LGBP on chitosan feeding and chitinases on β-glucan feeding, suggest that these immune-relevant proteins in *M. rosenbergii* are possibly multifunctional and might possess alternate modes of induction for synthesis; that needs further in-depth characterization.

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Authors' contribution: J. Mohanty, B.B. Patnaik and P.K. Sahoo: Designed the project, participated in the coordination and management during the study and edited the manuscript; **D. Panda and B.R. Pillai:** Did all animal experiments and edited the manuscript; **K.C. Das:** Did the feed formulation and preparation; **S. Sahoo, M.R. Badhe, A. Paul and S. Baliarsingh:** Performed all other experiments and wrote the main manuscript text. All authors read and approved the final manuscript.

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Conflict of interest: The authors declare no competing interests.

Data availability: Not applicable.

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