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Effectiveness of fermented and non-fermented *Chara corallina* on growth performance and digestive enzyme activities in Giant Freshwater Prawn (*Macrobrachium rosenbergii* de Man 1879)

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Abstract Results showed the average daily growth, specific growth rate, feed conversion ratio, protein efficiency ratio, and survival rate were not significantly different among the treatments ($p>0.05$). The highest survival rate was observed in the prawn fed a diet containing 30.0 % fermented *Chara corallina* added. Non-fermented *C. corallina* at 7.5% and 15.0% were significantly increased protease and cellulase activities, while both fermented and non-fermented forms led to a reduction in amylase activity. No significant changes were observed in lipase activity across all supplementation levels and forms. There was no significant interaction between algal fermention and inclusion levels on growth and survival rate ($p>0.05$). However, a significant interaction was found for protease, amylase and cellulase of freshwater prawn ($p<0.05$). According to this study, it can be concluded that *C. corallina* has the potential to substitute soybean meal protein in the diet of *M. rosenbergii*.

Keywords: Brittle wort, Freshwater prawn, Digestive enzyme, Growth, Algal fermentation

Introduction

Macrobrachium rosenbergii De Man (1879), the giant freshwater prawn, farming is known to be the largest in Asia. The giant freshwater prawn is an economically significant aquatic species in Thailand, particularly in the central

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region (FAO, 2011). It is widely consumed both within the country and abroad. The giant prawn culture is a crucial industry in Thailand, valued for the prawns good taste and high nutritional content. However, the industry still encounters challenges related to growth, disease, and the need for continued research and development in prawn feed. Therefore, enhancing the immune system of prawns is one approach to disease prevention. In recent years, various supplements have been tested in prawn feed, such as using of probiotics to increase the production of giant river prawns such as *Schizochytrium* sp. for farming all-male giant prawns (Sisouvong *et al*., 2015; Seenivasan *et al*., 2016), sea lettuce (*Ulva rigida*), green caviar (*Caulerpa lentillifera*), and red seaweed (*Acanthophora spicifera*) as feed additives (Aiemsomboon *et al*., 2021), fermented seaweed, *Kappaphycus alvarzii* in diets of juvenile freshwater prawn ; using fermented and non-fermented *Ulva lactuca* seaweed (Felix and Brindo, 2014), *Chlorella vulgaris* for growth and immune response (Maliwat *et al*., 2021).

Freshwater macroalgae utilization in aquaculture to promote growth or replace fishmeal and, as well as to improve aquatic health, has been studied and applied for a long time. Examples include *Spirulina* (*Arthrospira*) and green algae (*Cladophora*), which have demonstrated high effectiveness in freshwater aquaculture. However, there are no research reports on the cultivation of edible local freshwater algae in the southern region of Thailand in an organic manner. Preliminary studies have found that *Chara corallina* has high nutritional value, particularly with a high protein content, chlorophyll and carotenoid, and high valuable of phytochemicals (Chankaew *et al*., 2020; 2024). However, only the young tips of *C. corallina* are consumed. Therefore, the remaining parts, including the old thallus which are black and some physical characteristics of the algae that are less desirable for consumption, can be utilized as feed ingredients for aquaculture.

Silage, which is made from fermented plants, breaks down cellulose and carbohydrates into smaller molecules, and proteins are digested into amino acids. Bacteria in the silage produce acetic acid, lactic acid and propionic acid, which can inhibit the growth of microorganisms. Fermentation also helps reduce nitrate content in plants. Fermentation of plants results in a decrease in ash, fiber, and anti-nutritional factors (Cruz *et al*., 2011). For the use of fermented algae as feed for aquatic animals, examples include: Using *Ecklonia maxima* to feed Red Sea bream, which can reduce viral infections (Uchida, 2007). Feeding pearl oysters (*Pinctada martensii*) with *Undaria pinnatifida* fermented with *Lactobacillus* bacteria (Uchida and Miyoshi, 2010). Feeding Seabass (*Lates calcarifer* Bloch, 1970) with *Microspora* fermented algae (Koedprang and Phumee, 2015).

At present, some aquaculture operators are increasingly producing their own feed, due to the rising cost of feed, high raw material prices and production

costs. Therefore, finding raw materials, particularly various types of aquatic plant or algae with suitable properties for freshwater prawn feed, is an important and interesting. These raw materials should be affordable and readily available locally to assist farmers in reducing the feed cost. Thus, *C. corallina* is potential raw material that could substitute soybean meal protein in giant freshwater prawn feed, it is cost-effective and accessible. The aim of this study was to add value the unused thallus as a raw material for giant freshwater prawn feed, to examine the growth performance, to find the digestive enzyme activity for a potential approach for replacing soybean protein in aquaculture.

Materials and methods

Experimental design

2 x 4 factorial experiment in a Completely Randomized Design (CRD), which factor A was fermented and non ferment and factor B was four levels of *C. corallina Cc* at 0, 7.5, 15.0 and 30.0 %with four replications.

Preparation of the experimental diets

C. corallina (*Cc*) was collected from Krabi province Thailand. The alga was cleaned until sediment and materials were completely removed and then dried under room temperature. The alga was ground into powder with an herb grinder and store at 4 ^{0}C for proximate analysis (AOAC, 2016). Eight experimental feeds were prepared, containing different of the powered of *Cc* both fermented and non–ferment and proximate composition of each experimental diet formula was set to 35 % protein and 6 % lipid. As shown in Table 1, experimental diets, consisted of fish meal, soybean meal, fermented *Cc*, rice bran, wheat flour, wheat gluten, soy oil, lecithin (60%), vitamin/min premix, choline chloride, NaCl, dicalcium phosphate, rice husk and varying concentrations of *Cc* powder. All powder ingredients were sieved using a 200 μm sieve, and the ingredients were thoroughly mixed. The diet was dried in an oven for 10-12 h at 55-60 ^{0} C until the moisture content reached approximately 1.85% and then stored in a refrigerator at 4^{0} C. The diet pellets from each formulation were subjected to proximate analysis. All feed ingredients were weighed, then the dry stuffs were mixed for 10 min, then the oil was added, and mixed again for 10-15 min. After that 30-35% of water was added and mixed homogenously about 10-15 min. The dough then put through the pelleting machine with 2.5 mm of pellet diameter and evenly cut. The pellets were then oven dried at 60 °C for 10-15 hr. After cooling down and sieving the dust, the

feed was stored in plastic bags and kept refrigerated at 4 °C. Nutrition compositions of experimental feeds were also analyzed.

Nutrition compositions of experimental feeds

Nutrition compositions of experimental feeds are presented in Table 1. The protein contents were closed to the designed levels (30.0%). There were no statistically significant differences in protein and fat contents of experimental feeds ($p>0.05$).

Experimental shrimp and feeding trial

The giant freshwater prawn was obtained from a private farm and acclimatized in fiber tanks. They were fed twice a day for 4 weeks. The experimental shrimp with average weight of 0.08±0 . 0 1 g. was randomly distributed into the 200 L plastic tanks with 100 L of water. The stocking number was 20 prawns/tank. They were fed with experimental feeds until satiation three times per day for 120 days. Aeration was supplied throughout the experimental period. Tank cleaning and 80% water exchange were done every other day.

Prawn behaviors, feeding behavior and other symptoms of shrimp during experimental period were observed. Growth and survival of experimental shrimp were measured and recorded every 30 days for 90 days. Other growth parameters were calculated as following formulas.

$$\text{Average daily growth (ADG; g.day}^{-1}\text{)} = \frac{\text{final weight - initial weight}}{\text{day}}$$

$$\text{Specific growth rate (SGR; \%.day}^{-1}\text{)} = \frac{\text{ln (final average weight) - ln (initial average weight) x 100}}{\text{day}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{total feed weight (g)}}{\text{prawn weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{weight gain (g)}}{\text{protein intake (g)}}$$

$$\text{Weight gain (g)} = \text{final average weight - initial average weight}$$

$$\text{Survival rate (\%)} = \frac{\text{final number prawn x 100}}{\text{initial number of prawn}}$$

Table 1. Ingredient quantities and proximate composition of experimental diets and proximate chemical compositions of giant freshwater prawn feeds

Ingredients ($g*100^{-1}$)	Experimental feeds						
	Control	7.5% *Cc*F	15% *Cc*F	30% *Cc*F	7.5% CcNF	15.0% *Cc*NF	30% *Cc*NF
fish meal	22.000	22.00	22.00	22.00	22.00	22.00	22.00
soybean meal	40.000	37.00	34.00	28.00	37.00	34.00	28.00
***Cc* fermented**	0.000	4.066	8.132	16.323	0.000	0.000	0.000
***Cc* non-fermented**	0.000	0.000	0.000	0.000	6.896	13.711	27.371
rice bran	10.000	10.00	10.00	10.00	10.00	10.00	6.00
wheat flour	8.20	8.20	8.20	8.20	8.20	8.20	4.62
wheat gluten	4.00	4.00	4.00	4.00	4.00	4.00	4.00
soy oil	2.835	2.859	2.884	2.957	2.873	2.912	3.205
Lecithin (60%)	1.500	1.500	1.500	1.500	1.500	1.500	1.500
chloresterol	0.100	0.100	0.100	0.100	0.100	0.100	0.100
vitamin / mineral premix	2.000	2.000	2.000	2.000	2.000	2.000	2.000
choline chloride	0.350	0.350	0.350	0.350	0.350	0.350	0.350
NaCl	0.500	0.500	0.500	0.500	0.500	0.500	0.500
dicalcium phosphate	0.350	0.350	0.350	0.350	0.350	0.350	0.350
rice husk	8.165	7.075	5.984	4.009	4.271	0.377	0.000
Crude protein	33.15 ±0.01[a]	34.00 ±0.00[a]	34.18 ±0.00[a]	34.65 ±0.00[a]	33.50 ±0.02[a]	33.06 ±0.06[a]	32.59 ±0.43[a]
Crude fat	5.84 ±0.00[a]	6.06 ±0.02[a]	5.60 ±0.10[a]	5.92 ±0.02[a]	5.95 ±0.06[a]	5.63 ±0.03[a]	6.46 ±0.00[a]
Crude fiber	6.71 ±0.01[a]	5.90 ±0.00[a]	6.32 ±0.01[a]	6.33 ±0.00[a]	5.40 ±0.06[a]	4.41 ±0.04[a]	14.68 ±0.02[a]
Moisture	8.95 ±0.00[a]	8.98 ±0.02[a]	9.66 ±0.01[a]	8.12 ±0.01[a]	9.47 ±0.01[a]	9.01 ±0.01[a]	9.33 ±0.01[a]
Ash	11.27± 0.05[a]	10.98 ±0.01[a]	11.86 ±0.06[a]	12.35 ±0.06[a]	11.46 ±0.01[a]	11.55 ±0.01[a]	13.41 ±0.06[a]

Note: values are the mean±SD of three replication: means within each row superscripted with different letters are significantly ($p<0.05$) different

Digestive enzyme extraction method

The method for digestive enzyme extraction was adapted from Kattakdad *et al*., (2020). Hepatopancreas samples from giant freshwater prawns were collected from each treatment group after 16 hours of feeding. Each sample was weighed and finely ground using a homogenizer in Tris–HCl buffer (50 mM, pH 7.5) at a 1:3 (w/v) ratio of sample weight to buffer volume. The homogenate was then centrifuged at 15,000 x g for 30 min at 4°C. The supernatant was collected and stored at –20°C for subsequent enzyme activity analysis.

Digestive enzyme activities analysis

Protease activity was measured using 2% casein as the substrate, following the method of Pan *et al*., (2005). Lipase activity was assessed using *p*-nitrophenyl palmitate (pNPP) as the substrate, according to the method of Markweg *et al*., (1955). Amylase activity was determined using 1% starch solution as the substrate, based on the method outlined by Bernfeld (1995). Cellulase activity was analyzed using 1% carboxymethyl cellulose (CMC) as the substrate, as described by Miller (1959). Enzyme activity is reported in units per milligram of protein. Protein concentration in the enzyme extracts was determined using Lowry's method (Lowry *et al.*, 1951) with bovine serum albumin used as the protein standard.

Statistical analysis

All data were verified for normality, and data from each treatment were subject to a two-way ANOVA. Differences between means were tested using Duncan's new multiple rang test (DMRT) at a 5% probability level.

Results

Growth performance of giant freshwater prawn

Growth performances and feed efficiency of giant freshwater prawn fed with experimental diets for 12 weeks were shown in Table 2. There were no statistical differences in average weight, average daily growth and specific growth rate of all treatments ($p>0.05$). The prawn fed with 30.0% fermented algal supplemented feed had the lowest FCR of 2.080 ±0.34, and the highest PER of 1.41±0.25 which no statistically significant differences ($p>0.05$) with the control group. No significant differences were found of both FCR and PER among the prawn fed 7.5%, 15.0% and 30.0% supplemented feeds and control group. However, the prawn fed supplemented feed at 30.0% of fermented algal

supplemented had the better FCR and PER than those of the control and the other groups. In addition, survival rate of giant freshwater prawn fed with 30.0% of fermented and non-fermented algal supplemented feeds were higher than those of the prawn in control group and 7.5%, 15.0% supplemented feeds but there were no statistical differences (p>0.05).

Table 2. Growth performances and feed efficiency of freshwater prawn fed with different levels of fermented and non fermented *C. corallina* inclusion feeds

Item	*Cc*F-*Cc*NF (A)	algal inclusion (B)				avg.effect of *Cc*F-*Cc*NF	p-value		
		0%	7.5%	15.0%	30.0%		A	B	AxB
average daily growth (g/prawn)	*Cc*F	0.011±0.00[a]	0.009±0.00[a]	0.008±0.00[a]	0.011±0.00[a]	0.010±0.00[a]	0.936	0.348	0.348
	*Cc*NF	0.011±0.00[a]	0.009±0.00[a]	0.008±0.00[a]	0.010±0.00[a]	0.010±0.00[a]			
	avg.effect of algal inclusion	0.011±0.00[a]	0.010±0.00[a]	0.008±0.00[a]	0.011±0.00[a]				
specific growth rate (%prawn /d)	*Cc*F	2.71±0.29[a]	2.67±0.34[a]	2.48±0.42[a]	2.82±0.19[a]	2.67±0.14[a]	0.854	0.721	0.843
	*Cc*NF	2.71±0.29[a]	2.64±0.09[a]	2.67±0.19[a]	2.78±0.32[a]	2.70±0.06[a]			
	avg.effect of algal inclusion	2.67±0.14[a]	2.66±0.02[a]	2.58±0.13[a]	2.80±0.03[a]				
FCR	*Cc*F	2.26±0.26[a]	2.38±0.42[a]	2.41±0.93[a]	2.80±0.34[a]	2.463±0.23[a]	0.184	0.051	0.591
	*Cc*NF	2.26±0.26[a]	2.41±0.09[a]	2.63±0.29[a]	2.52±0.38[a]	2.455±0.16[a]			
	avg.effect of algal inclusion	2.26±0.01[a]	2.40±0.02[a]	2.52±0.15[a]	2.66±0.19[a]				
PER	*Cc*F	1.35±0.17[a]	1.25±0.20[a]	1.23±0.14[a]	1.41±0.25[a]	1.311±0.85[a]	0.362	0.408	0.818
	*Cc*NF	1.35±0.17[a]	1.24±0.05[a]	1.15±0.13[a]	1.24±0.19[a]	1.245±0.08[a]			
	avg.effect of algal inclusion	1.35±0.15[a]	1.25±0.13[a]	1.19±0.12[a]	1.32±0.22[a]				
survival rate **(%)**	*Cc*F	73.33±7.64[a]	76.67±5.77[a]	78.33±7.64[a]	80.91±8.66[a]	77.31±3.18[a]	0.887	0.458	0.998
	*Cc*NF	73.33±7.64[a]	76.64±7.63[a]	76.67±5.77[a]	80.00±5.01[a]	76.66±2.72[a]			
	avg.effect of algal inclusion	73.33±6.83[a]	76.69±6.06[a]	77.05±6.12[a]	80.00±6.24[a]				

Note: Values are the mean± standard deviation (SD) of triplicates, the same superscript letters in rows denote statistical difference (p<0.05); A= fermented and non fermented *Cc*, B=algal inclusion levels; AxB = interaction between fermented and non fermented *Cc* and algal inclusion levels in experimental diet

Digestive enzyme activities in giant freshwater prawn

The study of digestive enzyme activities in giant freshwater prawns after receiving experimental diets, it was observed that both the form (fermented, non-ferment) and the level of *Cc* supplementation influenced the activities of protease, amylase, and cellulase (p<0.05), but did not affect lipase activity (p>0.05). Supplementation with non-fermented *Cc* at 7.5% and 15.0% levels stimulated protease enzyme activity more effectively than the control group (p<0.05). However, high levels of non-fermented *Cc* 30.0% decreased protease enzyme activity. Similarly, fermented *Cc* supplementation led to reduced protease enzyme activity (p<0.05). In terms of amylase activity, fermented and non-fermented *Cc* supplements resulted in lower amylase activity compared to

the control ($p<0.05$). On the other hand, *Cc* supplementation enhanced cellulase enzyme activity relative to the control group ($p<0.05$). Among the treatments, 15.0% non-fermented *Cc* supplementation produced the highest cellulase enzyme activity ($p<0.05$). Nevertheless, *Cc* supplementation did not affect lipase enzyme activity in the digestive tract of giant freshwater prawns ($p>0.05$) (Table 3).

Table 3. The protease, lipase, amylase and cellulase specific activity in digestive tract of giant freshwater prawns at optimum pH

Item	*Cc*F - *Cc*NF (A)	algal inclusion (B)				avg.effect of *Cc*F-*Cc*NF	p-value		
		0%	7.5%	15.0%	30.0%		A	B	Ax B
protease (U/mg protein)	*Cc*F	0.087±0.006^{a}	0.067±0.004^{b}	0.056±0.003^{c}	0.050±0.004^{c}	0.065±0.016^{a}	0.00	0.00	0.00
	*Cc*NF	0.087±0.006^{a}	0.091±0.003^{a}	0.091±0.009^{a}	0.074±0.001^{b}	0.086±0.008^{b}			
	avg.effect of algal inclusion	0.087±0.005^{a}	0.079±0.013^{a}	0.074±0.020^{a}	0.062±0.013^{b}				
lipase (U/mg protein)	*Cc*F	361.75±17.43^{a}	309.49±27.24^{a}	327.28±28.80^{a}	302.52±31.90^{a}	325.26±26.466^{a}	0.638	0.042	0.947
	*Cc*NF	361.75±17.43^{a}	326.96±35.96^{a}	339.16±42.93^{a}	299.79±53.84^{a}	331.915±25.810^{a}			
	avg.effect of algal inclusion	361.75±15.59^{a}	318.23±30.09^{a}	333.29±33.34^{a}	301.15±39.61^{a}				
amylase (U/mg protein)	*Cc*F	0.530±0.003^{a}	0.522±0.006^{b}	0.485±0.004^{c}	0.486±0.003^{c}	0.506±0.024^{a}	0.00	0.00	0.00
	CcNF	0.530±0.003^{a}	0.502±0.002^{c}	0.490±0.004^{C}	0.470±0.003^{d}	0.498±0.022^{b}			
	avg.effect of algal inclusion	0.530±0.002^{a}	0.512±0.004^{a}	0.487±0.004^{b}	0.478±0.009^{b}				
cellulase (U/mg protein)	*Cc*F	0.035±0.005^{d}	0.040±0.001^{bc}	0.041±0.000^{b}	0.041±0.000^{b}	0.039±0.002^{a}	0.165	0.00	0.00
	CcNF	0.035±0.005^{d}	0.041±0.001^{b}	0.045±0.001^{a}	0.039±0.001^{d}	0.040±0.004^{a}			
	avg.effect of algal inclusion	0.035±0.000^{b}	0.040±0.001^{a}	0.043±0.002^{a}	0.040±0.001^{a}				

Note: Values are the mean± standard deviation (SD) of triplicates, the same superscript letters in rows denote statistical difference ($p<0.05$); A= fermented and non fermented *Cc*, B=algal inclusion levels; AxB = interaction between fermented and non fermented *Cc* and algal inclusion levels in experimental diet

Discussion

Growth performances of giant freshwater prawn

The giant freshwater prawn fed with *Cc* fermented and non fermented at different levels supplemented feeds at 7.5, 15.0, and 30.0 % for 90 days, there were no statistical differences of average daily growth, specific growth rate,

FCR, PER and survival rate of all treatments ($p>0.05$), it could be concluded that at 30.0 % of *Cc* fermented, FCR and PER of the prawn tended to the best, with the low FCR and the high survival rate and PER, and better the control group. Considerable increment in the protein and lipid contents of the fermented *Cc* (protein and lipid from 19.41% to 32.71% and, 1.22 to 0.85, respectively) was recorded. Microbial protein is believed to contribute significant to the protein content of fermented product. During fermentation, an increase in the nutrient level through microbial (Wee, 1991). Similar of *Cc* fermentation this study which using yeast that improvement of nutritional value of fermented algal that crude protein higher than non-fermented. The *Cc* fermented of fiber contents showed drastic reduction 14.74 % to 7.38%. The increasing level of raw and fermented of *Cc* resulted in growth and feed efficiency no significance from control group with used soybean meal replace protein in the diet. This results, concluded the growth of prawn tended to increase the level of raw *Cc* and fermented up to 30 % due to FCR, PER and survival rate is better than those of the control. The freshwater prawn fed in all level inclusion up to 7.5-30.0% of *Cc* did not affect the growth and survival rate. The similar results had been reported by Wattanakul *et al*. (2012) that a study on the growth and survival rate of freshwater prawn fed with supplemental fish steamed water with contained 35% protein and supplemental 50-300 g/kg feed with protein 34.55-40.06 %. The FCR of all replacement groups in this study were lower than diet supplemental fish steamed water with contained 35% protein and supplemental 50-300 g/kg feed and protein replacement of fish meal by mackerel condensate in diet (Wattanakul *et al*., 2012; Wattanakul *et al*., 2017). The optimal performance of freshwater prawn in terms of ADG, SGR, FCR, PER and survival rate was found in the diets containing 30.0% fermented *Cc*. than non fermented *Cc* and control group. This result concluded that, the soybean meal protein can be replaced in diet of *M. rosenbergii* with both the *Cc* fermented and unfermented. In addition, supplementation of *Cc* all level can promote growth of giant freshwater prawn was not affected on survival rate.

Digestive enzyme activities of giant freshwater prawn

The study on digestive enzyme activities found that supplementation with non-fermented *Cc,* a green macroalgae*,* stimulates the activity of protease and cellulase enzymes, particularly the synthesis of cellulase by intestinal microorganisms (Harris, 1993). These findings are consistent with Mustafa and Nagakawa (1995), which reported that algae can promote protein absorption and nutrient utilization. The relationship between digestive enzymes and feeding habits has been highlighted in several studies, demonstrating that digestive

enzymes play a critical role in determining the digestibility and utilization of various feed components (Glass and Stark, 1995; Johnston and Freeman, 2005; Castro *et al*., 2012). Furthermore, the results are consistent with Domozych *et al*. (2010), who described *Cc*, a charophycean green algae, as possessing cell walls rich in polymers similar to those found in land plants, including cellulose, pectic polymers and arabinogalactan proteins. Similarly, Furne *et al*. (2005) emphasized that digestion and nutrient absorption depend on the availability and efficiency of digestive enzymes. In crustaceans, enzyme activity is influenced by various factors including dietary composition, ontogenetic changes, and the relative size of the midgut during differentiation. These factors subsequently influence growth and molting cycles (Lovett and Felder, 1990). The supplementation of *Cc* produced similar effects similar to microalgae supplementation in shrimp diets. Anand *et al*. (2013) reporting microalgae supplementation in *Penaeus monodon* diets stimulated the growth of beneficial microorganisms in the digestive tract and the production of digestive enzymes. However, the supplementation of both fermented and non-fermented *Cc* did not affect lipase activity and had a negative effect on amylase activity in freshwater prawns, which contrasts with the findings of Radhakrishnan *et al*. (2016), who reported that supplementing 50% microalgae (*Arthrospira platensis*) in freshwater prawn diets stimulated both amylase and lipase activities. Lee *et al*. (2009) quantified the digestive enzymes in freshwater prawns, demonstrating their omnivorous nature and ability to efficiently utilize both plant-based feeds. Additionally, amylase activity has been shown to vary during the ontogenetic development of prawn larvae (Qing and Xing, 1997). Besides the type of feed, identifying and characterizing digestive enzymes is crucial at different grow-out stages (Celis-Guerrero *et al*., 2004). Thus, *Cc* supplementation presents a viable alternative for formulating feeds that enhance digestive enzyme activities of giant freshwater prawns.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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