Pakistan J. Zool., vol. 58(3), pp 1239-1246, 2026 DOI: https://dx.doi.org/10.17582/journal.pjz/20240809060256

Effect of Dietary Supplementation of Calcium Carbonate Nanoparticles on Growth, Survival and Immune Response of White Leg Shrimp (*Litopenaeus vannamei)*

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Article Information
Received 09 August 2024
Revised 25 January 2025
Accepted 19 February 2025
Available online 21 August 2025 (early access)
Published 31 March 2026

Authors' Contribution
LN conducted the study and wrote original manuscript. CR, AP and KM planned the work and supervised the research. CHL edited manuscript. RMK, SSV and KB results analysis. NMS and RSS helped in final drafting.

Key words
***Litopenaeus vannamei*, Nanoparticles, Calcium carbonate, Growth, Immune response**

ABSTRACT

A 63-day study was conducted to examine the effects of calcium carbonate nanoparticles on the growth and immune response of white leg shrimp, *Litopenaeus vannamei*. The shrimp were fed with four experimental diets containing increasing concentrations of calcium carbonate nanoparticles: 25 mg kg^{-1} (T_1), 50 mg kg^{-1} (T_2), 75 mg kg^{-1} (T_3), and 100 mg kg^{-1} (T_4), along with a control diet without nanoparticles. The T_2 (50 mg kg^{-1}) treatment exhibited significantly higher ($p>0.05$) final weight (10.09±0.07 g), specific growth rate (3.61±0.04), survival rate (91%) and low FCR (1.42±0.06)compared to the other treatments and the control. There was also a significant increase ($p>0.05$) in total haemocyte count (42.36±2.69), serum protein levels (79.35±1.13), and superoxide dismutase activity (6.9±0.08) inT_2 group compared to T_1, T_3, T_4, and the control. The findings of the study suggest that *L. vannamei* supplemented with 50 mg kg^{-1}calcium carbonate nanoparticles could enhance growth and health status.

INTRODUCTION

The shrimp *Litopenaeus vannamei* is a prominent candidate species in aquaculture, accounting for 80% of global shrimp production (Wyban, 2019). Total fisheries and aquaculture production reached an all-time record of 214 million tonnes in 2020, comprising 178 million tonnes of aquatic animals and 36 million tonnes of algae, a slight increase from the previous 2018 record of 213 million tonnes (FAO, 2022). The majority of our knowledge regarding the impact of dietary nutrients on shrimp health is derived from nutrients like minerals (Lin *et al*., 2013). Among which, calcium is a vital nutrient for both aquatic plants and animals, playing a crucial role in calcification necessary for hardening the cuticle. Calcium is an essential element in the body that is required for bone formation, growth, cellular physiology, immune response, and blood coagulation (Reid *et al*., 1993). Additionally, calcium is essential for maintaining physiological homeostasis and molting cycle. Generally, calcium is absorbed from food and discarded cuticle (Li and Cheng, 2012). $CaCO_3$ is most commonly used as calcium supplements (NRC, 1994). Additionally, it also affects the biological activity of crustaceans, including growth and reproduction,

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0030-9923/2026/0003-1239 $ 9.00/0

which are commonly influenced by water hardness (Mente, 2003; Houng *et al*., 2010). Especially for shrimp, calcium is not only a major component of the exoskeleton (Venkataramaiah *et al*., 1978) but also plays a role in osmoregulation (Shewbart *et al*., 1973), making it a critical mineral for shrimp. Crushing calcium carbonate to the nanoscale can significantly increase the rate of calcium absorption and utilization (Huang *et al*., 2015). Additionally, using nanomaterials for delivery systems can improve the nutritional profiles of feed and feeding conversion rate (Bhattacharyya *et al*., 2015).

Due to its easy availability and slow biodegradability, $CaCO_3$ is used in controlled drug delivery and drug encapsulation, including for bioactive proteins in pharmaceuticals (Garg *et al*., 2021). The studies on the effect of dietary calcium on water quality, growth, survival and immune response of shrimp has been documented to some extent (Cheng *et al*., 2006; Furtado *et al*., 2014; Andrews *et al*., 1973). However, there was a very limited information on the effects of $CaCO_3$ nanoparticles on the basic physiology and biochemistry of crustaceans (Srinivasan *et al*., 2017). Thus, as per my knowledge this was the first study on the effect of calcium carbonate nanoparticles on the growth and immune response of *L. vannamei*.

MATERIALS AND METHODS

Acclimatization

The healthy *Litopenaeus vannamei* post-larvae (PL-12) were obtained from the CP (Charoen Pokphand) Hatchery in Nellore District, Andhra Pradesh, India, and transported to the Aquaculture Wet Laboratory at College of Fishery Science, Muthukur under minimum stress. Upon arrival, the post-larvae were acclimatized in circular FRP (fiber reinforced polymer) tanks. The tanks were continuously aerated, and the shrimp were properly fed for 20 days with commercial feed with 35 percent protein content. During this acclimation period, water quality parameters were maintained at optimal levels: pH 7.10 ± 0.20, dissolved oxygen 6.20 ± 0.36 mg L^{-1}, and ammonia 0.018 ± 0.004 mg L^{-1} and salinity 15 ppt.

Feed formulation

All ingredients shown in Table I were weighed, ground into a powder, mixed as 100g with 30 ml of water per 100 g of feed were added to form a dough, with refined wheat flour (1%) included as a binding agent. The dough was cooked under pressure for 20 min, then allowed to cool before adding vitamin premix, calcium-free mineral premix, and fish oil.

The $CaCo_3$ nanoparticles (<100 nm particle size, 99.99% purity, 2.7 g/cm³ density, 100.08 g/mol molecular weight) procured from Nanoshel Nanomaterial Global Chemical Supplier, Punjab were incorporated into the basal diet at four different concentrations *viz*., 25, 50, 75, and 100 mg kg^{-1}. The dough was processed using a pelletizer (La Monferrina S.R.L., Italy) with a 1 mm diameter sieve to produce uniform pellets. The pellets were dried in the shade for 2-3 days and in a hot air oven at 80–90°Cto reduce moisture content to 10%. The prepared feed was stored in airtight containers within a desiccator until use in the feeding trials.

Table I. Feed formulation of the diets ingredients (g/100g).

Treatments	C	T_1	T_2	T_3	T_4
Fish meal	20	20	20	20	20
SBM	20	20	20	20	20
Shrimp meal	10	10	10	10	10
GNOC	10	10	10	10	10
DOB	26	26	26	26	26
Maize	8	7.9975	7.995	7.9925	7.99
Maida	2	2	2	2	2
Vit-premix	1	1	1	1	1
Min-premix	1	1	1	1	1
Fish oil	1	1	1	1	1
Soya lecithin	1	1	1	1	1
Nano Ca	0	0.0025	0.005	0.0075	0.01

SBM, soya bean meal; GNOC, groundnut oil cake; DOB, deoiled rice bran

The proximate composition of the feed was determined using AOAC (1995) methods. Moisture content was measured using a hot air oven at 105 °C. The crude protein content was assessed using the Kjeldahl method. Ether extract was determined with a Soxhlet apparatus, and ash content was measured using a muffle furnace at 600 °C for 6 h. The defatted and moisture-free sample was treated sequentially with diluted acid (1.25%) and alkali to estimate crude fiber content.

Experimental design and feeding

The experiment was conducted over 63 days in rectangular plastic aquarium tanks measuring 60×30×30 cm, each filled with 50 liters of water. The study included four treatment groups and one control group (without calcium carbonate). The treatment tanks were set up with increasing concentrations of calcium carbonate as follows: T_1(25 $mgkg^{-1}$), T_2 (50 mgkg-^{1}), T_3 (75 $mgkg^{-1}$), and T_4 (100 $mgkg^{-1}$). Each tank was stocked with 15 shrimp (average weight of 1.25±0.04 g) and had three replicates. To ensure adequate oxygen levels, all tanks were well-aerated using

three aeration pipes. Throughout the experiment, the shrimp were fed four times daily (6 AM, 10 AM, 2 PM, and 6 PM) at a rate of 5% of their body weight. Additionally, 10% fresh seawater was added to all tanks regularly to maintain good water quality.

Water quality parameters

During the study period, water pH (universal pH indicator), dissolved oxygen (Aqua check DO test kit, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and temperature (Mercury bulb thermometer) were measured daily at 10:00 AM. Total hardness and alkalinity (Water Testing kit, Nice Chemicals Pvt, Ltd, Kochi, India) were measured once in every three days. Salinity was measured with portable refractometer (ERMA, RHS-28) once in a week while adding freshwater to compensate evaporation loss.

Growth and survival

The shrimps in all the experimental tanks were sampled at weekly intervals and weighed using an electronic balance(Mettler-Toledo GmbH, Switzerland). The growth performance and survival rate were assessed on weekly basis. The growth parameters such as final weight, specific growth rate, net weight gain and food conversion ratio and survival rate was calculated by using the following formulae of Panigrahi *et al*. (2018).

Immunological parameters

At the end of the experiment, shrimp haemolymph was collected from each tank. Fifteen shrimp per treatment group (five per replicate) were anesthetized with clove oil (50 µl/l). Haemolymph was drawn from the ventral sinus using a 26-gauge needle and a 2-ml syringe with anticoagulant solution (30 mM tri-sodium citrate, 388 mM sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55). The haemolymph was mixed with an equal volume of pre-cooled anticoagulant, pooled from each tank, stirred gently in a sterile centrifuge tube, and stored on ice for further analysis.

For serum collection, haemolymph was collected from five additional shrimp per treatment into centrifuge tubes without anticoagulant, kept at 4ºC for 6 h, and then centrifuged at 10,000 rpm for 30 min. The bluish supernatant (serum) was stored at -80ºC for future use.

Haemato-immune parameters

For hematology, a drop of anticoagulant-mixed haemolymph (1:1 ratio) was placed on a Neubauer hemocytometer and observed under an Olympus light microscope at 400x magnification to count the total haemocyte count (THC) (Wootton and Pipe, 2003). After counting total haemocytes, they were categorized into granulocytes and agranulocytes (hyaline cells) based on the granular content (Le Moullac *et al*., 1997) and expressed as total granulocyte cells ml^{-1} (TGC ml^{-1}) and total agranulocyte cells count (TAC ml^{-1}). The THC, TGC and TAC were calculated by the following formulae of Wootton and Pipe (2003).

Total serum protein and SOD activity

Total protein concentration in serum samples was measured using the Bradford Method (1976). Superoxide dismutase (SOD) activity was measured using a Sigma-Aldrich SOD determination kit, following the manufacturer's instructions.

Statistical design and analysis

Statistical analysis was done with SPSS (version 2.0). All the growth and immune parameters were statistically analyzed using one-way ANOVA at 5% significance level, as per the standard statistical methods by means of Duncan multiple range test.

RESULTS

Feed formulations

Table II shows the proximate composotion of ingredients used for formulating experminatl diet.

The proximate composition of expermintal feed was found to be: protein 35.5%, moisture 9.35%; ether extract 5.85%, fiber 5.30% and ash 5.95% water quality.

Table II. Proximate composition of ingredients (as % dry basis) used for experimental diets.

Ingredients	Fishmeal	Soya meal	Shrimp meal	GNOC	DOB	Maize
Crude protein	60.0 ± 0.406	45.35± 1.34	50.23± 1.535	46.14± 1.369	15.33± 0.571	10.18± 0.321
Crude fiber	3.53± 0.082	8.42± 0.143	5.71± 0.126	11.40± 0.143	14.23± 0.205	3.75± 0.076
Moisture	7.24± 0.068	9.62± 0.096	9.43± 0.115	10.29± 0.162	9.29± 0.116	8.95± 0.06
Ether extract	3.82± 0.179	1.62± 0.461	4.45± 0.062	1.59± 0.055	2.43± 0.203	1.67± 0.504
Total ash	14.91± 0.289	9.38± 0.186	24.65± 0.11	9.25± 0.149	8.79± 0.105	2.27± 0.148
NFE	17.71	35.23	14.96	31.62	59.22	82.13

NFE, nitrogen free extract; For other abbreviation, see Table I.

The water parameters, including temperature, pH, total alkalinity, hardness, and dissolved oxygen, were within the acceptable range for *Litopenaeus vannamei* in all treatment tanks, including the control and the ranges are shown in Table III.

Growth and survival

All the growth parameters and survival rates of shrimp in all experimental tanks are presented in Table IV. Growth parameters such as weight gain and specific growth rate (SGR) were significantly lower ($p>0.05$) in the control tank compared to the treatment tanks. Among the four treatments, shrimp in the T_2, which were fed 50 mg/kg calcium carbonate nanoparticles, exhibited the highest weight gain (10.09±0.07) and SGR (3.61±0.04). The food conversion ratio (1.42±0.06) was significantly lower ($p<0.05$)in the T_2 when compared to the other three treatments and the control tank (1.81±0.06).

During the initial three weeks of the experiment, the survival rate of *L. vannamei* was 100% across all treatments. The significantly highest ($p > 0.05$) survival rate was observed in T_2 (91.11%), while the lowest was recorded in control (64.44±1.67). Further, there was no significant differences were found in the survival rates between the T_1 and T_3, which were fed 25 mg/kg and 75 mg/kg calcium carbonate nanoparticles, respectively. Moreover, only 77% survival rate was recorded in T_4 which was fed with 100 mg/kg of calcium carbonate.

Haemato-immune parameters

The shrimp in control showed significantly low haemato-immunological response when compared to treatments. Among the treatments, T_2 showed significantly higher hematological performance such as THC, TGC, TAC and immunological response including serum protein, SOD. The haemato-immunological parameters of the shrimp in all the experimental tanks are given in Table V.

Table III. Water quality parameters of shrimps fed with calcium carbonate nanoparticles.

Parameters	Control	T_1	T_2	T_3	T_4
Temperature	29.2± 0.17	29.0± 0.25	29.0± 0.06	30.1± 0.15	29.2± 0.32
DO	6.80± 0.152	7.10± 0.251	7.32± 0.1	7.34± 0.115	6.63± 0.12
pH	8.3± 0.153	8.3± 0.252	8.3± 0.1	8.1± 0.115	8.2± 0.153
Alkalinity	152± 2.65	162± 3.79	160± 3.21	168± 3.51	154± 4.51
Hardness	620± 4.73	680± 4.04	660± 2.52	690± 3.51	650± 3.51

DO, dissolved oxygen

Table IV. Nutritional indices of *L. vannamei* fed on calcium carbonate nanoparticles.

Parameters	Control	Dietary $CaCo_3$ NPs (mg kg^{-1})			
		T_1	T_2	T_3	T_4
Initial weight	1.25±0.03^a	1.25±0.04^a	1.25±0.09^a	1.25±0.03^a	1.25±0.12^a
Final weight	7.69±0.08^a	9.05±0.07^d	10.09±0.07^e	8.14±0.07^c	7.8±0.07^b
Weight gain	0.79±0.06^a	0.96±0.04^b	1.14±0.05^d	1.03±0.02^c	1.01±0.04^c
Survival rate	64.44±1.67^a	80±1.26^c	91.11±1.05^d	80±1.18^c	77.77±2.04^b
SGR	2.88±0.02^a	3.14±0.03^c	3.61±0.04^d	2.97±0.02^b	2.94±0.04^b
FCR	1.81±0.06^d	1.52±0.03^b	1.42±0.06^a	1.58±0.04^c	1.55±0.02^b

Note: Means ±SD at 0.05 level of significance by using Duncan's test in SPSS. SGR, specific growth rate; FCR, food conversion ration.

Table V. Immune parameters of *L. vannamei* fed on calcium carbonate nanoparticles.

Parameters	Control	Dietary $CaCo_3$ NPs (mg kg^{-1})			
		T_1	T_2	T_3	T_4
Total haemocyte count (THC) (x10^6 cells ml^{-1})	27.29±1.58^a	38.10±2.32^d	42.36±2.69^e	32.24±3.67^c	28.23±2.24^b
Total hyaline cell count (x10^6 cells ml^{-1})	7.73±0.79^a	12.41±1.21d	16.53±1.63^e	11.21±1.52^c	8.71±1.19^b
Total granulocyte count (TGC) (x10^6 cells ml^{-1})	15.35±1.84^a	21.28±2.42^d	25.32±2.89^e	19.19±2.60^c	17.34±1.68^b
Serum protein (mg/ml)	63.04±1.26^a	76.67±1.44^d	79.35±1.13^e	72.39±1.09^c	67.13±1.72^b
Superoxide dismutase (SOD) activity (µ/mol)	3.6±0.03^a	6.2±0.02^c	6.9±0.08^d	6.0±0.05^c	4.7±0.03^b

Note: Means ±SD at 0.05 level of significance by using Duncan's test in SPSS.

DISCUSSION

Aquatic animals need minerals as essential components of their diets. These include calcium, phosphorus, magnesium, potassium, copper, iron, zinc, manganese, selenium, and iodine. Especially in fish, calcium is one of the most abundant cation and is vital for bone development and maintaining skeletal structure. It is also widely distributed in soft tissues and plays a key role in cell membrane integrity, nerve signal transmission, muscle contraction, blood clotting, and enzyme activation. Additionally, calcium is tightly bound to phospholipids in cell membranes, affecting membrane permeability and nutrient absorption (Lall, 2002). Similarly, crustaceans such as penaeid shrimp and lobsters require minerals like calcium, copper, magnesium, phosphorus, potassium, selenium, and zinc (Davis and Gatlin, 1996). Among these minerals, calcium is crucial for their growth because it aids in the calcification of their cuticle (Li and Cheng, 2012).

Water quality parameters

Water quality parameters in all experimental groups were suitable for *L. vannamei* (Van *et al*., 1999; Lin and Chen, 2003). Chen *et al*. (1985) stated that the best DO level for shrimp growth and survival is 5 mg/L. In the present study, we have recorded DO in between the range of 5.30-5.70 mg/L from all the experimental tanks. Further, no significant difference was found in water parameters including temperature, DO, pH, alkalinity and hardness among the treatments and control.

Growth parameters

Calcium is a crucial mineral for essential physiological functions like molting and growth development in crustaceans (Luo *et al*., 2013). Cheng *et al*. (2006) observed a higher growth rate in *L. vannamei* fed with 1% dietary calcium. Similarly, Furtado *et al*. (2014) found that the 10% and 20% calcium supplemented treatments resulted in superior growth rates, while the lowest growth was observed in the 40% supplemented treatment, indicating that higher calcium concentrations negatively impact growth. Recently, Fakhari *et al*. (2020) reported that prawn *Macrobrachium nipponense* showed accelerated growth when consuming calcium carbonate nanoparticles (25–50 mg/kg) with these particles being stored in the hepatopancreas. In the present study, *L. vannamei* fed with 50 mg/kg calcium carbonate (T_2) exhibited significantly higher average weight, weight gain, and specific growth rate, suggesting that proper mineralization supports shrimp growth through effective molting. However, increasing the calcium carbonate concentration from 50 mg/kg to 100 mg/kg was found to potentially reduce growth rates, likely due to antagonistic effects on the absorption of other minerals. This aligns with findings by Andrews *et al*. (1973) demonstrated that increasing calcium levels from 1.5% to 1.75% or 2.0% resulted in a noticeable drop in fish weight gain. Additionally, the current study's findings are consistent with Fakhari *et al*. (2020) also observed a decrease in growth rate and survival of *M. nipponense* when calcium levels increased from 50 mg/kg. In this study, *L. vannamei* had the lowest feed conversion ratio (FCR) in the T_2 tank (1.42) compared to other treatments and the control (1.81), which is similar to Furtado *et al*. (2014) who reported the lowest FCR values in 10% and 20% treated tanks compared to 40%.

The highest survival rate was recorded in the T_2 group (91%), followed by T_3 (80%), T_1 (80%), and T_4 (77%). These results are in line with Furtado *et al*. (2014), who found a decreasing survival trend from 10% (91.76) to 20% (93.72) and 40% (85.03). Although we observed a lower growth rate in the T_3 treatment, the survival rate was still above 80%, suggesting that supplementing shrimp with 75 mg/kg of calcium carbonate is ineffective in promoting growth but had no effect on animal survival. Further lowest survival was recorded in T_4 treatment which was fed on 100 mg/kg of calcium carbonate, which indicating that high level of calcium carbonate nanoparticles may negatively affect the animal (Fakhari *et al*., 2020).

Immune response

Shrimp has nonspecific innate immune response and a very limited memory (Sarathi *et al*., 2007). Components of hemolymph have been frequently employed as metrics to track the immunological, nutritional, and physiological status of crustaceans under different stress conditions (Matozzo *et al*., 2011; Porchas *et al*., 2011). Hence, nanoparticles (size up to 100 nm) have drawn attention as immunostimulants, drug delivery systems, and antimicrobials (Shaalan *et al*., 2016; Swain *et al*., 2014).

Generally, the total haemocyte count (THC) in penaeid shrimps ranges from 20-40 x 10^6 cells ml^{-1} of haemolymph (Chang *et al*., 1999; Kumar *et al*., 2015). In the present study, *L. vannamei* shrimp of T_2 treatment showed significantly high THC, (42.36 x 10^6 cells ml^{-1}), total hyaline cells (16.53 x 10^6 cells ml^{-1}), total granulocyte count (25.32 x 10^6 cells ml^{-1}), serum protein (79.35mg/ml) and SOD (6.5µ/mol). Further, we found decrease in haemocyte count, SOD and serum protein in other treatment shrimps which was fed on higher levels of calcium carbonates. These findings were in accordance with Muralisankar *et al*. (2014) who also reported a decreasing trend in haemocyte count in *M. rosenbergii* with increasing zinc nanoparticles supplementation. Which suggests that, by increasing the inclusion level of nanoparticles may decrease the

immune response of the animal. Serum proteins such as hemocyanin and other enzymes give the serum lytic and defensive qualities in crustaceans. Shrimp that consumes a larger percentage of natural food have higher amounts of haemolymph metabolites, which include protein, albumin, glucose, triglycerides, and cholesterol. These metabolites are an indicator of the nutritional status of the shrimp (Gong *et al*., 2000). In crustaceans, the haemocyanin makes up 90–95% of the serum protein concentration (Depledge and Bjeregaard, 1989) and so, its decrease may have an impact on that animal's particular immune proteins (Perazzolo *et al*., 2002). Hence, findings of the present study demonstrating that more than 50 mg/kg of calcium carbonate may show toxic effect on the shrimp.

CONCLUSION

This study concludes that calcium carbonate nanoparticles are an effective mineral component for enhancing the growth and immune performance of *L. vannamei*. The findings clearly shows that shrimp fed with 50 mg/kg of calcium carbonate nanoparticles exhibited significantly higher growth, survival, and immune response. Additionally, the study also reveals that increasing the concentration from 50 to 100 mg/kg decreases growth, survival, and immune response.

DECLARATION

Acknowledgement

First author heartfully acknowledges the Andhra Pradesh Fisheries University (APFU) for providing fellowship and laboratory facilities in the Department.

Funding

The study received no external funding.

IRB approval

Ethical approval was obtained from the Institutional Review Board (IRB) of Andhra Pradesh Fisheries University.

Ethical approval

The animals used in this study were handled very carefully and study was approved by A.P.F.U Committee, 2023.

Statement of conflict of interest

The authors have declared no conflict of interest.

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