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The effect of dietary sodium alginate on immune reactions and resistance against *Aeromonas hydrophila* in giant freshwater prawn (*Macrobrachium rosenbergii*)

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Abstract

The present study evaluated the effects of dietary sodium alginate on immune responses and disease resistance against *Aeromonas hydrophila* in giant freshwater prawn (*Macrobrachium rosenbergii*). Juveniles were fed diets supplemented with sodium alginate at 0 (control), 1.0, 2.0, and 3.0 g/kg feed for 60 days. Immune parameters including phenoloxidase activity, respiratory burst activity, and total hemocyte count were assessed after the feeding trial. Prawns fed sodium alginate exhibited significantly enhanced immune responses compared with the control group. The highest values of phenoloxidase activity (0.977 ± 0.006), respiratory burst activity (0.112 ± 0.002), and total hemocyte count ($10.392 \pm 0.101 \times 10^6$ cells/ml) were observed in the 2.0 g/kg treatment. A challenge test with *A. hydrophila* revealed significantly lower mortality ($35.00 \pm 2.89\%$) and higher relative percent survival ($51.78 \pm 3.42\%$) in the same treatment group. These findings indicate that dietary sodium alginate at 2.0 g/kg effectively enhances immunity and improves resistance against bacterial infection in *M. rosenbergii*.

Keywords: *Macrobrachium rosenbergii*, Sodium alginate, Immunostimulant, Phenoloxidase activity, Respiratory burst activity, Total hemocyte count, *Aeromonas hydrophila*, Disease resistance, Relative percent survival, Aquaculture

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is a candidate culturable freshwater prawn species with a high growth rate, euryhaline, wide temperature tolerance ($15-35^\circ\text{C}$), and larger size. In India, freshwater prawns are mostly cultivated in mono- and polyculture systems in either naturally occurring or controlled bodies of water (Mukhopadhyay *et al.*, 2003) [1]. The strong demand for this prawn is due to its high nutritional value, including its high levels of protein, polyunsaturated fatty acids, and low-fat levels (D'Abramo & Sheen, 1994) [2]. But, apart from these prawns, the major constraint in prawn farming is the high rate of mortality due to higher rate of stocking, algal bloom production, DO depletion, man-made food polluted water, and pathogen diseases (Farook *et al.*, 2019) [3].

The use of antibiotics in aquaculture, especially during larval and growth stages, has raised concerns about environmental and human health impacts, such as bacterial resistance, persistent aquatic diseases, and altered sediment biogeochemistry (Bermúdez-Almada & Espinosa-Plascencia, 2012; Ma *et al.*, 2006) [4, 5]. Excessive antibiotic use promotes antimicrobial-resistant bacteria (AMRB) and fosters antibiotic resistance mechanisms (Pruden *et al.*, 2013) [6]. To address these challenges, environmentally friendly alternatives like probiotics, immunostimulants, antimicrobial peptides, and quorum sensing interference have been developed (Sivakamavalli *et al.*, 2021) [7]. Probiotics and prebiotics have gained attention as more cost-effective solutions compared to antibiotics or vaccines, with prebiotics offering the advantage of being natural feed ingredients that promote beneficial gut bacteria and enhance health (Gatesoupe, 2005; Yousefian & Amiri, 2009) [8, 9].

Additionally, medicinal plants, such as marine algae, show promise as natural prebiotics due to their bioactive, antibacterial properties that are safe for the environment and beneficial in aquaculture (Alghazeer *et al.*, 2013; Dashtiannasab *et al.*, 2016) ^[10, 11]. Algal extracts can be delivered to prawns through injection, immersion, or feed supplementation (Huang *et al.*, 2006; Yeh *et al.*, 2006) ^[12, 13].

Sodium alginate, a natural hydrophilic polysaccharide derived from marine brown algae, is composed of 1,4-d-mannuronic (M) and l-guluronic (G) acids and serves as a structural component in algal cell walls, containing 30-60% alginic acid (Maiti & Kumari, 2016; Santos, 2017) ^[14, 15]. Widely used in aquaculture as a feed additive, it functions as a feed binder to improve pellet stability, a dietary fiber, and a prebiotic promoting gut health in herbivorous and omnivorous fish species. Sodium alginate enhances the intestinal absorption of minerals like calcium and magnesium (Mohapatra *et al.*, 2011) ^[16] and is recognized as GRAS (generally regarded as safe) by the FDA (Stone *et al.*, 2009) ^[17]. Known for its biocompatibility, nontoxicity, and non-immunogenicity, it also possesses antibacterial, antiviral, antimicrobial, antifungal, antioxidant, immunomodulatory, and anti-inflammatory properties (Sachan *et al.*, 2015; Ngo & Kim, 2013; Guo *et al.*, 2020) ^[18, 19, 28]. Additionally, alginic acid exhibits hypocholesterolemic and antihypertensive effects. Its potential for immunostimulatory and resistance studies, such as in *Macrobrachium rosenbergii* against *Aeromonas hydrophila*, highlights its versatile applications in aquaculture.

2. Material & Methods

2.1 Sodium Alginate

Sodium alginate, which was purchased from Isochem Laboratories in Kochi, Kerala.

2.2 Experimental Diet

The commercial diet was cooked, sterilized, and powdered. Then, powdered Sodium alginate was added to diets in four doses, including control (T₀), 1.00 g/kg (T₁), 2.00 g/kg (T₂), and 3.00 g/kg (T₃). After that, the dough was thoroughly mixed to distribute evenly all the ingredients. Then prepare pellets, and spread them into trays for sun drying. After drying, pellets were packed into airtight plastic containers, labeled, and stored properly.

2.3 Experimental Animal & Husbandry Trial

The experiment was conducted at the Hands-on Training Center, Department of Aquaculture, situated within the College of Fisheries Science, Kamdhenu University, Veraval. *Macrobrachium rosenbergii* juveniles (~0.2 g) were obtained from Dev Fisheries in Valsad, 695 km from the experimental site. Packaged in 15-liter plastic bags with treated freshwater and pure oxygen, sealed bags (1,000 juveniles per bag) were transported in thermocol boxes to minimize stress during transit. Upon arrival, the juveniles underwent prophylactic treatment in a 0.05% potassium permanganate solution following the normal bath method (Cruz-Lacierda *et al.*, 2000) ^[23] and were transferred to a 500-liter tank for acclimatization. During the 10-day acclimation period, they were fed *Artemia* (live feed) and commercial feed, with continuous aeration to maintain

optimal oxygen levels before being moved to experimental tanks.

The experiment was conducted in 16 plastic tanks (2 x 1 x 1 feet, 40 liters), disinfected with 2 ppm KMnO₄ and rinsed thoroughly. The tanks were divided into four treatment groups with four replicates each. A total of 240 *Macrobrachium rosenbergii* juveniles (~0.2 g initial weight) were acclimatized and randomly distributed (15 prawns per tank) following a completely randomized design (CRD). The prawns were cultured for 60 days, and fed experimental diets twice daily initially at 10% of body weight for the first month, then reduced to 5% thereafter. Feedings occurred from 9:00-9:30 a.m. and 6:30-7:00 p.m. Weight was monitored biweekly, and feeding rations were adjusted accordingly.

2.4 Immune Parameter Analysis

Following the culture period, immune parameters in prawns from all four experimental groups were assessed by analyzing phenoloxidase activity, respiratory burst activity, and total hemocyte count. Hemolymph was collected by anesthetizing the prawns and extracting it directly from the heart using a 1 ml insulin syringe (26-gauge) preloaded with 100 µl of anticoagulant solution (0.114 M trisodium citrate and 0.1 M sodium chloride, pH 7.45) to prevent clotting. The hemolymph was transferred to an Eppendorf tube, with additional anticoagulant added to maintain the required ratio. Phenoloxidase activity was determined using the prophenoloxidase assay (López *et al.*, 2003) ^[21] by incubating hemolymph with trypsin (0.1 mg/ml) and L-DOPA (3 mg/ml in Cac buffer), followed by measuring the absorbance at 490 nm to indicate dopachrome formation. Respiratory burst activity was measured using the nitroblue tetrazolium (NBT) assay (Song and Hsieh, 1994) by incubating hemolymph in microtiter plates at 37°C to allow cell adherence, adding NBT solution, washing with 70% methanol, and dissolving formazan with potassium hydroxide and dimethyl sulfoxide (DMSO), with optical density (OD) recorded at 620 nm using an ELISA reader. Total hemocyte count was determined with a Neubauer chamber hemocytometer under a light microscope, following the method described by Sritunyalucksana *et al.* (2005) ^[23].

2.5 Resistance study

A resistance test was conducted with 40 *Macrobrachium rosenbergii* juveniles from each treatment after the culture period, with 10 prawns stocked per tank and injected with *Aeromonas hydrophila* in triplicates, while 10 prawns raised individually received PBS injections as negative controls. The *A. hydrophila* strain, purchased from Hi-media Pvt. Ltd., was cultured and preserved using *Aeromonas hydrophila* isolation medium, then incubated in tryptic soy broth (TSB) at 30°C for 24 hours. Bacterial pellets were extracted by centrifuging the broth at 7000 rpm for 15 minutes at 4°C, washed with PBS, and suspended to an optical density (OD) of 1.00 at 600 nm, equivalent to 1 × 10⁸ CFU/ml, and serially diluted to 10⁷ CFU/ml. Prawns were injected intramuscularly with 0.1 ml of *A. hydrophila* suspension (10⁷ CFU/ml) or PBS (for controls) using a 1 ml insulin syringe (26-gauge) between the second and third abdominal segments. Dead prawns were removed, and clinical signs were monitored for seven days. Resistance was evaluated using mortality percentage and relative

percent survival (RPS), calculated as $RPS = [1 - (\text{Mortality\% in treatment group} / \text{Mortality\% in control group})] \times 100$, with mortality recorded every 24 hours for seven days post-injection.

2.6 Statistical Analysis

After the experiment, data was evaluated using conventional statistical techniques by Snedecor and Cochran (2014). The analysis was carried out using the SPSS program. To assess "Mean $\pm$ Standard Error (SE)," one-way analysis of variance (ANOVA) was utilized; to test for significant differences across treatments, the Duncan multiple range test (DMRT) was employed.

3. Results and Discussion

3.1 Immune Parameters Analysis

3.1.1 Phenoloxidase activity

The outcomes of phenoloxidase (PO) activity levels in *Macrobrachium rosenbergii* across different treatments are presented in Table and Figure 1. Prawns fed a sodium alginate-incorporated diet showed significantly higher PO activity compared to those fed a fish meal-based control diet ($P < 0.05$). The highest activity was observed in treatment T₂ ($0.977 \pm 0.006a$), while the lowest was recorded in the control group (T₀, $0.607 \pm 0.004d$). The melanization process, regulated by the prophenoloxidase activation cascade, plays a vital role in crustacean immunity by responding to pathogen invasion (Amparyup *et al.*, 2013)^[24]. Enhanced PO activity in sodium alginate-fed prawns may result from its antimicrobial properties against pathogens (Ngo & Kim, 2013; Lee *et al.*, 2011)^[19, 25]. Among the treatments, the 2.0 g/kg sodium alginate diet (T₂) showed the highest activity, followed by 1.0 g/kg and 3.0 g/kg diets, with the control diet exhibiting the lowest activity. Similar findings were reported by Yudiati *et al.* (2016)^[26] and Cheng *et al.* (2005)^[27], where sodium alginate diets increased PO activity in shrimp species.

3.1.2 Respiratory burst activity

The results of respiratory burst (RB) activity, presented in Table and Figure 2, highlight superoxide anion production in prawns. Sodium alginate-fed prawns exhibited significantly higher RB activity compared to those fed the control diet ($P < 0.05$). The highest RB activity was observed in treatment T₂ ($0.112 \pm 0.002a$), where prawns were fed a 2.0 g/kg sodium alginate diet, while the lowest was recorded in the control group ($0.090 \pm 0.001d$). Respiratory burst, a metabolic process in immune cells like phagocytes, generates reactive oxygen species (ROS) to combat pathogens. However, ROS can also cause cellular damage, necessitating the role of antioxidant enzymes like superoxide dismutase (SOD) in neutralizing ROS (Yudiati *et al.*, 2016)^[26]. The study suggests that sodium alginate enhances RB activity by increasing NADPH oxidase activity and SOD levels, as observed in *M. rosenbergii* fed sodium alginate diets. Similar findings were reported by Cheng *et al.* (2005)^[27] and Liu *et al.* (2006)^[28], where sodium alginate at 2.0 g/kg improved RB and SOD activities in shrimp, demonstrating a strengthened immune response.

3.1.3 Total hemocyte count

The Total Hemocyte Count (THC) results, presented in Table and Figure 3, show the highest THC in treatment T₂ ($10.392 \pm 0.101a$), followed by T₁ ($8.75 \pm 0.082b$), T₃ ($7.792 \pm 0.077c$), and T₀ ($6.497 \pm 0.046d$). Statistical analysis revealed significant differences ($P < 0.05$) among all

treatments, with prawns fed a 2.0 g/kg sodium alginate diet (T₂) exhibiting the highest THC, while the control group (T₀) had the lowest. Sodium alginate positively impacted THC by enhancing phagocytic activity and the phagocytic index in *M. rosenbergii*. Hemocytes transitioned from non-adhesive to adhesive cells upon recognizing foreign particles, forming a multilayered sheath around intruders during encapsulation (Yudiati *et al.*, 2016)^[26]. Similar findings by Yudiati *et al.* (2016)^[26] and Cheng *et al.* (2005)^[27] demonstrated that a 2.0 g/kg sodium alginate diet significantly increased THC compared to other treatments and the control diet.

3.2 Challenge Study

After the feeding trial, *M. rosenbergii* underwent a 7-day resistance test against *Aeromonas hydrophila* using the injection method, where bacteria was injected into the body of the host, *M. rosenbergii*. The control group (T₀) and treatment groups (T₁, T₂, T₃) received an intramuscular injection of 0.1 ml of *A. hydrophila* suspension, while the negative control group received an injection of 0.1 ml of PBS solution. Subsequently, the data was analyzed by counting the dead prawns in each group at 24 hour intervals following injection for a maximum of seven days (Figure 4). The results of the resistance study against *Aeromonas hydrophila* in *M. rosenbergii* are presented in Table 1 and Figure 4. The statistical analysis showed that the highest mortality was found in control at 72.50 ± 2.50^a . The T₂ treatment shows significantly lower mortality than all treatments. Hence, statistically, it is proven that dietary supplementation of sodium alginate can show resistance against *Aeromonas hydrophila* infection due to its antibacterial properties (Ngo & Kim, 2013; Lee *et al.*, 2011)^[19, 25]. The higher resistance is shown in T₂ (35.00 ± 2.89^c), followed by T₁ (42.5 ± 2.50^c), and T₃ (57.50 ± 2.50^b). The RPS of all treatments compared with control was also analyzed, yielding the following results: T₁: (41.07 ± 4.49^a), T₂: (51.78 ± 3.42^a), and T₃: (20.09 ± 5.80^b). The identical results were also obtained by Neamat-Allah *et al.* (2019), where an increase in sodium alginate concentration in the diet of catfish proportionally increases the resistance against *Aeromonas hydrophila* infection due to antibacterial properties of sodium alginate. Similarly, the result obtained by Cheng *et al.* (2005)^[27] increases the resistance of *L. vannamei* against *Vibrio* infection due to dietary administration of sodium alginate. The study conducted by Cheng *et al.* (2004)^[30] shows that an increase in phagocytic activity and respiratory burst activity of shrimp due to dietary administration of sodium alginate can be responsible for resistance activity against *Vibrio spp.* infection in shrimp, *L. vannamei*.

4. Conclusion

This study demonstrated the significant immunomodulatory potential of sodium alginate as a dietary supplement for enhancing the health and resistance of *Macrobrachium rosenbergii*. Among the tested treatments, the 2.0 g/kg sodium alginate supplementation (T₂) consistently yielded superior results across immune parameters, including phenoloxidase activity, respiratory burst activity, and total hemocyte count. These findings underscore sodium alginate's ability to strengthen the prawn's immune system, likely due to its antimicrobial, antioxidant, and immunostimulatory properties.

Moreover, the resistance study against *Aeromonas hydrophila* revealed that dietary sodium alginate

significantly reduced mortality rates and improved Relative Percent Survival (RPS), further supporting its role as an effective natural immunostimulant in aquaculture. Treatment T₂ exhibited the lowest mortality and highest RPS, suggesting that 2.0 g/kg is the optimal concentration for boosting disease resistance in *M. rosenbergii*.

Overall, the incorporation of sodium alginate as a feed additive offers a promising, eco-friendly alternative to

antibiotics in aquaculture. By enhancing immune response and resistance to pathogens, sodium alginate contributes to sustainable prawn farming practices, reducing reliance on chemical treatments while ensuring animal health and environmental safety. Future studies could explore its effects on other aquaculture species and evaluate long-term impacts on growth performance and water quality.

List of tables

Table 1: Phenoloxidase activity (PO) of *M. rosenbergii* fed with different concentrations of sodium alginate at the end of the experiment (Mean $\pm$ SE, n = 4)

Replication	Treatment			
	T ₀	T ₁	T ₂	T ₃
I	0.597	0.806	0.955	0.716
II	0.609	0.822	0.974	0.731
III	0.615	0.83	0.984	0.738
IV	0.607	0.819	0.971	0.729
MEAN $\pm$ SE (PO _{OD490nm})	0.607 $\pm$ 0.004 ^d	0.819 $\pm$ 0.005 ^b	0.9771 $\pm$ 0.006 ^a	0.728 $\pm$ 0.005 ^c

Table 2: Respiratory Burst (RB) of *M. rosenbergii* fed with different concentrations of sodium alginate at the end of the experiment (Mean $\pm$ SE, n = 4)

Replication	Treatment			
	T ₀	T ₁	T ₂	T ₃
I	0.089	0.103	0.111	0.096
II	0.091	0.107	0.114	0.098
III	0.093	0.108	0.116	0.101
IV	0.087	0.101	0.108	0.094
MEAN $\pm$ SE	0.090 $\pm$ 0.001 ^d	0.105 $\pm$ 0.002 ^b	0.112 $\pm$ 0.002 ^a	0.097 $\pm$ 0.002 ^c

Table 3: Total Hemocyte Count (THC) of *M. rosenbergii* fed with different concentrations of sodium alginate at the end of the experiment (Mean $\pm$ SE, n = 4)

Replication	Treatment			
	T ₀	T ₁	T ₂	T ₃
I	6.58	8.83	10.52	7.89
II	6.34	8.55	10.14	7.6
III	6.62	8.93	10.59	7.94
IV	6.45	8.7	10.32	7.74
MEAN $\pm$ SE	6.497 $\pm$ 0.064 ^d	8.75 $\pm$ 0.082 ^b	10.392 $\pm$ 0.101 ^a	7.792 $\pm$ 0.077 ^c

Table 4: Mortality rate (%) and Relative percent survival (RPS) of *M. rosenbergii* juveniles recorded in experimental groups after *A. hydrophila* injection (Mean $\pm$ SE, n=4)

Parameters	Treatments			
	T ₀	T ₁	T ₂	T ₃
No. of dead prawns	29/40	17/40	14/40	23/40
Mortality (%)	72.50 $\pm$ 2.50 ^a	42.5 $\pm$ 2.50 ^c	35.00 $\pm$ 2.89 ^c	57.50 $\pm$ 2.50 ^b
RPS against control	-	41.07 $\pm$ 4.49 ^a	51.78 $\pm$ 3.42 ^a	20.09 $\pm$ 5.80 ^b

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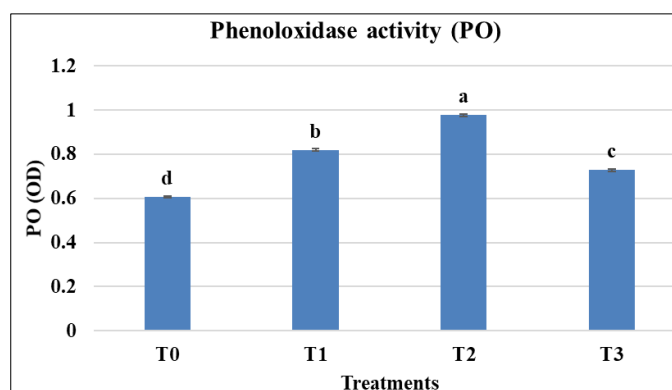


Fig 1: Impact of sodium alginate diet on *M. rosenbergii* phenoloxidase activity (PO) after the experiment
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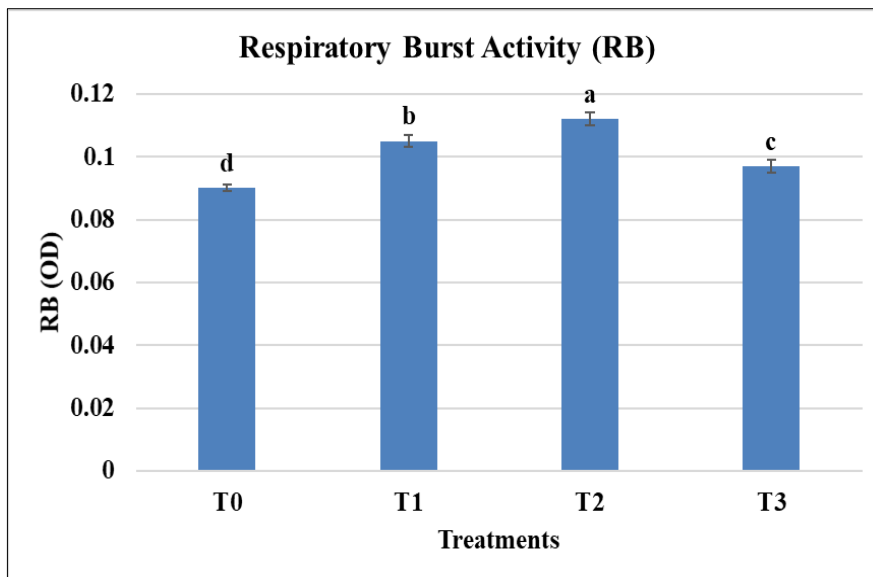


Fig 2: Impact of sodium alginate diet on *M. rosenbergii* respiratory burst activity (RB) after the experiment

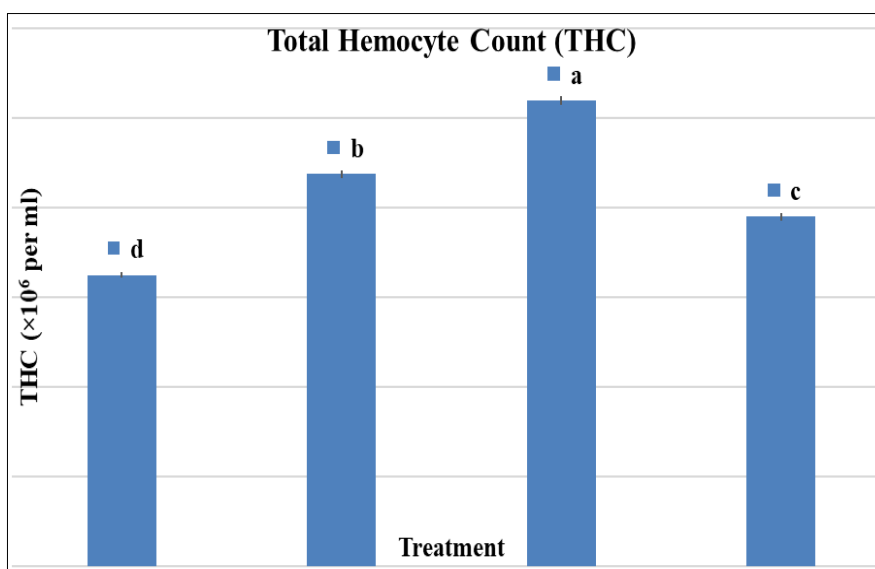


Fig 3: Impact of sodium alginate diet on *M. rosenbergii* total hemocyte count (THC) after the experiment

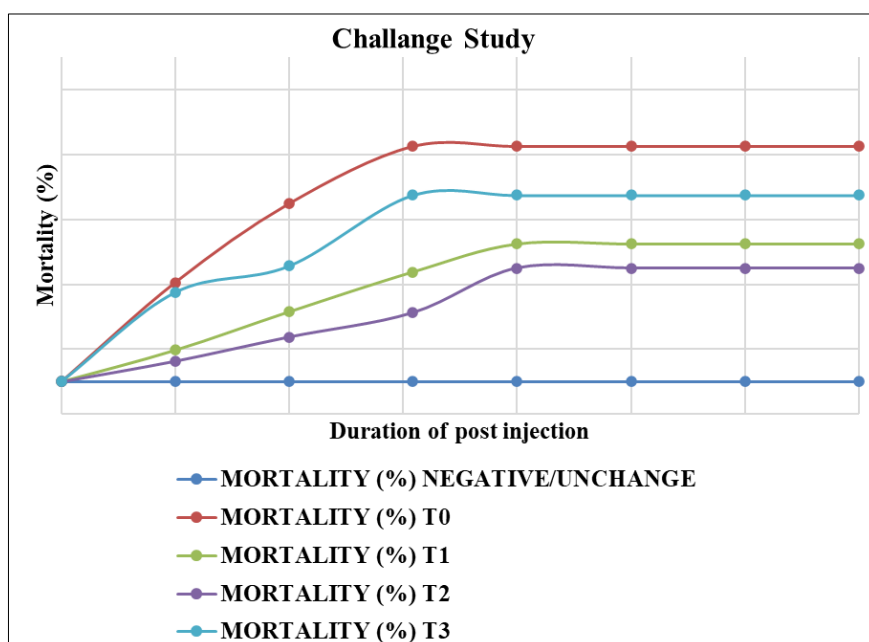


Fig 4: Impact of sodium alginate diet on mortality rate (%) of *M. rosenbergii* juveniles against *A. hydrophila* infection

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