Efficacy of probiotics, prebiotics, and immunostimulant on growth performance and immunological parameters of *Procambarus clarkii* juveniles

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- Immunostimulant;
- Growth;
- Immunity;
- *Procambarus clarkii* juveniles

**Abstract** *Procambarus clarkii* juveniles were used as an aquatic model to investigate growth performance and immune parameters after 6 weeks of feeding with supplementation of ten experimental diets containing 1%, 2%, 3% Biogen® (as probiotics), *Allium sativum* (garlic) and *Cynodon dactylon* (as immunostimulant) and one concentration (3 g/L) of sodium alginate (as prebiotics), compared with feeding on control basal diets only. All supplementation diets increased survival rates and wet weight, while, 1% and 2% *C. dactylon* showed a significant (*P* ≤ 0.05) increase in weight gain percentage (WG%), and specific growth rate (SGR). Feeding with diet containing 2% Biogen®, 2% and 3% garlic and sodium alginate showed a significant (*P* ≤ 0.05) increase in SGR rate after 6 weeks. Synergetic effect of 1% and 3% Biogen® and sodium alginate in total hemocytes count (THC) which increased significantly (*P* ≤ 0.05) after 6 weeks of feeding. Prophenoloxidase activity increased with all supplemented diets. While SOD increased significantly with 3% *C. dactylon*, 1% and 3% garlic and 2% Biogen. The results indicated that Biogen®, garlic, *C. dactylon* and sodium alginate inclusion with a basal diet had the potential to improve the growth and immune response of *P. clarkii* juveniles, hence this enables us to use the three supplementation diets in fish and prawn farms to improve their growth and immune parameters.

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**Introduction**

Aquaculture has a long history that can be traced back for more than 2000 years (Boyd and Tucker, 1998). Under intensive production systems, fish and crustaceans are exposed to various stressful conditions leading to growth reduction, immunosuppression and susceptibility to infectious diseases resulting in a major economic loss for farmers. The potential for reducing stress and enhancing immunity and disease resistance by nutritional factors/feed additives such as probiotics/prebiotics, immunostimulants and bacterins has been demonstrated in warm-blooded animals, fishes and crustacea.
During the past 50 years, numerous trials were conducted with microorganisms known as probiotics in efforts to improve culturability of food species, and to improve human health and welfare. Several probiotic species were used, including *Lactobacillus* spp, *Saccharomyces* sp, *Bacillus* spp, and mixed cultures (Muralidhara et al., 1977; Ozawa et al., 1981; Lessard and Brisson, 1987; Surawicz et al., 1989). The use of immunostimulants is being introduced into fish farming routine procedures as a prophylactic measure. These substances haven’t any negative side effects that live vaccines and antibiotics may have on consumers and on the environment, and are generally classified as biological response modifiers (Anderson, 1992; Secombes, 1994).

Probiotics are dietary supplements containing potentially beneficial live bacteria, yeast or algae, which when consumed in adequate amounts confer a health benefit for the host (FAO/WHO, 2001). Immunostimulants such as garlic (*Allium sativum*) and bermuda grass (*Cynodon dactylon*) increase the immune responses in several shrimp species in aquaculture by promoting phagocytosis, bactericidal activity, proPO activity, and respiratory bursts, and enhancing resistance against pathogens (Chang et al., 2000). Garlic increases fish welfare, and is able to support the control of pathogens, especially bacteria and fungi (Corzo-Martínez et al., 2007). *C. dactylon* inclusion with a basal diet had the potential to improve the growth and immune response of prawns *Macrobrachium rosenbergii* (El-Desouky et al., 2012). Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of a limited number of bacteria, usually bifidobacteria and lactobacilli, and thus improve host health (Gibson and Roberfroid, 1995). Growth performance and immunological parameters could be increased on using of probiotics, prebiotics and immunostimulants as daily supplementary diets (Cheng et al., 2005; Ziaei-Nejad et al., 2006; Diab et al., 2008; Ndong and Fall, 2011; El-Desouky et al., 2012; Poongodi et al., 2012).

*Procambarus clarkii* (the red swamp crayfish), is widely used in aquaculture and represents as an important food source for several vertebrate species. In addition to being a vector of the crayfish plague, due to its short life-history, rapid growth, burrowing activities and high population density, it can adversely impact the aquatic environment (Gherardi, 2006; Gherardi and Acquistapace, 2007).

Supplementary diet administration of Biogen® as probiotics, Garlic and *C. dactylon* as immunostimulant and sodium alginate as probiotic, was evaluated on growth performance every week for 6 weeks and immunological parameters as Total hemocytes count (THC). Prophenoloxidase (proPO) and Superoxide dismutase activity (SOD) of *P. clarkii* juveniles after 6 weeks feeding trial.

### Materials and methods

**Experimental animals**

330 Juveniles of *P. clarkii*, were obtained by size from brackish water in EL-Fayoum governorates, Egypt. Crayfish were housed in glass containers (60 L capacity), for 2 weeks before the beginning of the experiment. After acclimatization, individuals were distributed randomly into 33 glass aquaria (30 L capacity) at an initial capacity of ten juveniles per aquarium (tank). The water was changed every two days, including faeces and remaining food, then, each aquarium was refilled to a fixed volume using stored (dechlorinated) and well-aerated freshwater. The air-conditioner was installed in the environment-controlled laboratory maintained at 25 °C.

**Experimental diets**

Biogen® is a commercial probiotic, and consists of *Bacillus licheniformis* and *Bacillus subtilis* (Diab et al., 2008), constants were as follows: *Allicin* (not less than 0.247 Mmol g⁻¹), *B. subtilis* Natto (not less than 6 × 10⁷ g⁻¹) and *High Unit Hydrolytic Enzyme* (not less than 3690 U g⁻¹). Powdery forms of Garlic, *C. dactylon*, and sodium alginate were used. Prebiotics as sodium alginate (Wang et al., 2006) is a polyuronic saccharide that is isolated from the cell walls of brown seaweed (Brownlee et al., 2005). Artificial basal diet was fish grower consists from wheat flour, cod liver oil (Universal Medicare Pvt. Ltd.) and vitamin premix (Vetsfarma Ltd.), which contains 20% carbohydrates; 41% proteins; 15% lipids and 9% ash.

### Table 1

<table>
<thead>
<tr>
<th>Weight</th>
<th>W0</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.55</td>
<td>6.82</td>
<td>6.63</td>
<td>6.9</td>
<td>7.07</td>
<td>7.05</td>
<td>7.5</td>
</tr>
<tr>
<td>Biogen 1%</td>
<td>7.04</td>
<td>7.36</td>
<td>7.4</td>
<td>7.97</td>
<td>8.10</td>
<td>8.8</td>
<td>8.85</td>
</tr>
<tr>
<td>Biogen 2%</td>
<td>5.79</td>
<td>5.8</td>
<td>5.8</td>
<td>6.4</td>
<td>6.95</td>
<td>7.16</td>
<td>7.24</td>
</tr>
<tr>
<td>Biogen 3%</td>
<td>8.08</td>
<td>8.23</td>
<td>8.16</td>
<td>8.5</td>
<td>8.22</td>
<td>8.48</td>
<td>8.3</td>
</tr>
<tr>
<td>Garlic 1%</td>
<td>7.54</td>
<td>7.67</td>
<td>8.36</td>
<td>8.41</td>
<td>8.71</td>
<td>8.49</td>
<td>8.51</td>
</tr>
<tr>
<td>Garlic 2%</td>
<td>5.65</td>
<td>5.85</td>
<td>6.23</td>
<td>6.62</td>
<td>6.5</td>
<td>8.85</td>
<td>8.65</td>
</tr>
<tr>
<td>Garlic 3%</td>
<td>3.72</td>
<td>4.57</td>
<td>4.91</td>
<td>4.45</td>
<td>4.53</td>
<td>4.44</td>
<td>4.65</td>
</tr>
<tr>
<td>Cysteine 1%</td>
<td>4.77</td>
<td>4.97</td>
<td>5.81</td>
<td>6.67</td>
<td>6.6</td>
<td>6.7</td>
<td>6.58</td>
</tr>
<tr>
<td>Cysteine 2%</td>
<td>3.91</td>
<td>3.89</td>
<td>5.01</td>
<td>5.44</td>
<td>5.24</td>
<td>5.72</td>
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<td>5.59</td>
<td>5.72</td>
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<td>Sodium alginate</td>
<td>3.72</td>
<td>4.57</td>
<td>4.91</td>
<td>4.45</td>
<td>4.53</td>
<td>4.44</td>
<td>4.65</td>
</tr>
</tbody>
</table>

*P* ≤ 0.05.
Diets preparation

Eleven experimental diets were formulated to contain different levels (1%, 2%, 3%) of Biogen®, A. sativum (garlic) powder, C. dactylon, sodium alginate (3 g/L) and basal diets. To prevent food dissolving, each mixture (9 gm) was added to sodium alginate solution (3 gm/L). After incubation at 37°C overnight, a solution of 10% calcium carbonate was spread over the food film.

Crayfish juveniles were randomly divided into eleven groups, ten juveniles in each group with three replicates; Groups 1, 2 and 3 fed on basal diet mixed with 1%, 2%, 3% of Biogen respectively. Groups 4, 5 and 6 fed on basal diet mixed with 1%, 2%, 3% Garlic. Groups 7, 8 and 9 fed on basal diet mixed with 1%, 2%, 3% C. dactylon. Group 10 fed on basal diet mixed with sodium alginate solution. Control group fed on basal diet only. Juveniles were fed once daily at 9:00 am. Daily feeding rate was about 6% of total body weight (g). Samples from each aquarium were weighed weekly and the amount of daily diet was adjusted accordingly. Percentages of mortality rates were measured weekly.

Growth parameters and survival rates

Body weight (g) of each crayfish was measured weekly, and specific growth parameters were measured (Sweilum, 2006) as follows:

1. Specific growth rate (SGR) = (ln Wt – ln W0)/T × 100 (ln = natural Logarithm; W0 = initial weight; Wt = final weight; and T = time in day).
2. Feed efficiency ratio (FER) = live weight gained (g)/dry feed given (g) × 100.
3. Weight gained (WG) = (final weight-initial weight)/initial weight × 100.

Survival rate (%SR) = NSC/Total number of Crayfishes × 100

where, NSC: number of survived crayfishes

Total haemocyte count (THC)

Haemolymph (1.5 ml) was withdrawn from the ventral sinus of five juveniles from each group each into a 3 ml sterile syringe containing 1.5 ml anticoagulant solution (Trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 M, pH 7). A drop of the anticoagulant-haemolymph mixture was placed on a haemocytometer to measure THC using an inverted phase-contrast microscope.

Hemocyte count = \( \frac{N \times 20 \times 10}{4} \)

where, N: number of haemocytes counted in 4 corners, 20: dilution factor, 10: area of each corner and 4: number of corners.
Prophenoloxidase (proPO) assay

Prophenoloxidase activity measured in the haemolymph of *P. clarkii* juveniles according to Beyer and Fridovich (1987). Enzyme activity was expressed as a change in absorbance at 420 nm per 3 min. from the following equation:

\[ \Delta OD = (\text{High read} - \text{Low read})/3 \text{ min} \]

Enzyme activity = \( \frac{\text{OD} / \text{min} \times \text{Final volume} \times 4 \times \text{Dilution factor}/\text{Sample volume}}{\text{Dilution factor}} \)

where, OD: optical density

Superoxide dismutase activity

SOD activity measurement in *P. clarkii* juveniles' haemolymph was done by using the standard method of Nishikimi et al. (1972).

Statistical analysis

Statistical t-test was used to compare control (basal diet feeding) and treated *P. clarkii* with Biogen, garlic, and *C. dactylon* for 6 weeks. Differences were considered statistically significant at \( P \leq 0.05 \).

Results

Growth parameters

The wet weight change

All feeding diets groups increased in wet weight after 6 weeks of feeding (Table 1). The wet weight of *P. clarkii* juveniles showed the significant increase \( (P \leq 0.05) \) in 1% Biogen and *C. dactylon* and 2% Biogen and garlic feeding groups with 1.44, 1.3, 1.45 and 1.2-folds increase, respectively compared with the control group (0.95-fold increase).

Specific growth weight (SGR) and feed efficiency ratio (FER)

Specific growth rate and feed efficiency ratio were calculated for different food additive groups after 6 weeks of feeding as shown in Figs. 1 and 2. SGR increased significantly \((P \leq 0.05)\) in groups fed on basal diet mixed with 2% biogen (0.565 ± 0.035), 2.3% garlic (0.5285 ± 0.0015 and 0.479 ± 0.021, respectively) and 1%, 2% *C. dactylon* (0.7675 ± 0.0025 and 0.6865 ± 0.0035, respectively), and sodium alginate (0.336 ± 0.1). FER was calculated for all different food additive groups after 6 weeks of feeding. From all groups, only the group of 1% *C. dactylon* fed group showed a significant increase \((P \leq 0.05)\) in FER (0.2555 ± 0.0055) after 6 weeks of feeding compared to the control group (0.2005 ± 0.0105). 2% *C. dactylon* feeding group showed an insignificant increase in FER (0.2285 ± 0.0015) compared to the control group (0.2005 ± 0.0105).

Percentage of weight gain (%WG)

As shown in figure 3, the groups treated with 1% and 2% *C. dactylon* showed the highest significant increase in %WG (37.975 ± 0.025 and 33.285 ± 0.0153, respectively) compared to the control group (14.55 ± 0.05) followed by the groups fed with both 2% Biogen and 3% garlic (24.985 ± 0.015 and 24.9 ± 0.1, respectively) after 6 weeks of feeding compared to the control group.

Percentages of survival rate (%SR)

The survival rate (%) of *P. clarkii* juveniles after feeding with different food additives groups for 6 weeks are shown in Fig. 4. The highest survival rates were obtained in groups fed on a diet containing 3% garlic (83.28 ± 0.2) followed by 3% *C. dactylon* (80.43 ± 0.4) compared with the control group which exhibited the lowest rate (40%). Groups fed with different concentrations of Biogen (1%, 2% and 3%) exhibited an equal survival rate (63.4%).

Immunological parameters

Total hemocytes count

Total hemocyte (THC) count is shown in Fig. 5. THC was significantly increased \((P \leq 0.05)\) in both 1% and 3% Biogen treated groups and reached to 6437.5 ± 618.71 and 6175 ± 813.17 cells/ml respectively and this ratio increased insignificantly \((P \geq 0.05)\) in the 2% Biogen treated group (4891.5 ± 789.83 cells/ml) compared to the control group (3300 ± 50). Interestingly, THC in the group fed on basal diet added to a solution of sodium alginate (3 gm/L), haemocyte

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Figure 3   Percentage of weight gain (%WG) of *P. clarkii* fed on diets containing different food additives and control diet after 6 weeks.
count significantly increased (6157.83 ± 224.61) \((P \leq 0.05)\) compared to the control group.

**Prophenol oxidase (pro PO) activity**

As shown in Fig. 6, all *P. clarkii* juveniles fed on different diets had higher pro PO activity than the control one. Pro PO exhibited the highest activity in garlic followed by *C. dactylon* than Biogen feeding juveniles compared with the control group.

**Superoxide dismutase activity (SOD)**

SOD activity showed the highest significant ratio \((P \leq 0.05)\) in *P. clarkii* juveniles fed in 3% *C. dactylon* containing diet (298.62 ± 1.3) compared with the control group (228.41 ± 1.5), followed by 1% Garlic (261.83 ± 1.1), 2% Biogen (259.42 ± 0.57) and 3% garlic (258.5 ± 1.4) diet supplements which also increased significantly \((P \leq 0.05)\) compared with control group. Meanwhile, low significant SOD activity was shown in 3% *C. dactylon*, 2% garlic and 1% Biogen diet supplement groups which reached to (127.65 ± 2.3, 149.08 ± 0.91 and 187.83 ± 2.1 unit/ml, respectively) All the remaining groups (3% Biogen, 1% *C. dactylon* and sodium alginate containing diet) decreased significantly \((P \leq 0.05)\) compared with the control (Fig 7).

**Discussion**

Recent results showed that, probiotics as Biogen 1% and 2% improved weight gain, specific growth rate (SGR) and feed efficiency ratio (FER) and hence the survival rate after 6 weeks when added to basal diet of *P. clarkii* juveniles. The improvement in growth parameters was due to the change in *Bacillus* bacteria ratio in the gut flora. Significant increase in specific growth rate (SGR) and feed efficiency ratio (FER) of *M. rosenbergii* were detected after the administration of Biogen (Saad et al., 2009). Moreover, using different types of probiotics increased the growth performance of Nile tilapia, rabbit fish and European perch (EL-Haroun et al., 2006). On the other hand, administration of 3% Biogen to basal diet of *P. clarkii* juveniles had no effect on weight gain, SGR and FER. These results agreed with the study of Chotikachinda et al. (2008) and Sun et al. (2011). Positive effects of Biogen administration in the daily diet may be due to the *Bacillus* bacteria found in Biogen that secrete a wide range of exoenzymes and the presence of probiotics might stimulate the production of endogenous enzymes by the freshwater crayfish (Wang, 2007). Furthermore, it may be due to the effect of the tested probiotics which improved absorption of nutrients and depressed harmful bacterial (Ghazalah et al., 2010).
Administration of 1% and 2% garlic (as an immunostimulant) concentration levels enhanced the growth and survival rate of juveniles after 6 weeks. While, 2% and 3% garlic levels added to basal diet improved weight gain and specific growth rate (SGR). These results were in accordance with the study of Javadzadeh et al. (2012) on *Litopenaeus vannamei* shrimp. Similar results were also shown in Nile Tilapia by Mahfouz et al. (2009), Aly and Mohamed (2010) who observed an increase in growth, %WG and SGR after feeding with different doses of garlic. Also, Nya and Austin (2009) noticed an increase in growth after feeding *Oncorhynchus mykiss* fish with garlic. In addition Talpur and Ikhwanuddin (2012) showed a higher growth rate and SGR after feeding *Lates calcarifer* with garlic. Guo et al. (2012) and Ayoola and Uzoamaka (2013) showed similar results on both *Epinephelus coioides* and African catfish. Improvement in growth performance may be due to the presence of allicin in garlic, which promotes the performance of intestinal flora, thereby improves digestion, with better utilization of energy leading to improved growth (Khalil et al., 2001). Also garlic may evoke food desire hence feed intake as well as digestibility. Therefore, SGR enhances and led to higher weight gain (Talpur and Ikhwanuddin, 2012).

Administration of 1% and 2% *C. dactylon* (as an immunostimulant) enhanced all the growth performance and survival rates of *P. clarkii* after 6 weeks. These results agreed with Kaleeswaran et al. (2011) who noticed an increased in growth performance and body composition of *Catla catla* after feeding with ethanolic extract of *C. dactylon*. Similar results were also shown by El-Desouky et al. (2012) and Yogeeswaran et al. (2012) who noticed an increase in %WG and SGR% in *M. rosenbergii* and *Penaeus monodon* after feeding with *C. dactylon*. These results may be attributed to the presence of essential amino acids in *C. dactylon* (Stewart, 1973). Also, it may be attributed to those herbals which are reported to stimulate the secretion of digestive enzymes that can result in improvement in digestibility, stimulating the appetite and increasing food consumption and efficiency. In addition, they shorten the feed transit time which might have a beneficial influence on digestive enzymes and could accelerate the overall digestive process. (Platel and Srinivasan, 2004). Furthermore, Sodium alginate caused an improvement in growth, weight gain and specific growth rate of *P. clarkii* juveniles after 6 weeks of administration. Interestingly, it improved the physiological regulation and health status (Chung et al., 2011). It also improved growth in both Atlantic cod *Gadus morhua* (Vollstad et al., 2006) and *P. monodon* (Chung et al., 2011).
Dietary administration of Biogen improved immune response of *P. clarkii* juveniles due to an increase in phagocytic activity of granulocytes under the effect of *Bacillus* (Itami et al., 1998). Also, probiotics may induce a higher hemocyte proliferation (Rodriguez et al., 2007) and it may be due to change in the microbial community of the specimen which led to better immune responses (Li et al., 2007). Interestingly, elevated number of total hemocytes of *P. clarkii* juveniles accelerated the maturation of hemocyte precursor cells in the hemopietic tissue followed by release of new cells into circulation (Sequeira et al., 1996). Synergistic effect of Biogen and sodium alginate induces THC elevation.

Using garlic and *C. dactylon* as immunostimulant caused a decrease in THC and elevation in pro Po activity. According to the findings of Aly and Mohamed (2010), Yezizam and Ergün (2012) and Kanani et al. (2014) regarding different fish species garlic could induce a defense reaction in Juveniles (Fazlolahzadeh et al., 2011). Garlic contains useful therapeutic components such as hydroxyl radicals and superoxide anion serves as a defence source against infections (Kim et al., 2001 and Yang et al., 1993) elucidate elevation in the level of pro PO and SOD after feeding with garlic supplementary diets in the present study. Also the mode of action of allinic in garlic may well include inhibition of cysteine protease, scavenging and trapping of free radicals (hydroxyl, superoxide anions and hydrogen peroxides) and initiation of the inhibition of thiol-containing protein in the cells of pathogens (Ankri et al., 1997 and Rabinkov et al., 1998). Garlic has traditional applications as an anti-infective agent against many bacteria (Res et al., 1993), fungi (Adetumbi et al., 1986) and viruses (Weber et al., 1992). The antibacterial properties of garlic clove homogenates are attributed to allicin.

The present study showed elevation in pro PO activity under the effect of *C. dactylon* levels and SOD activity (only with 3% *C. dactylon*). These results agreed with Balasubramanian et al. (2008) and Yogeeswaran et al. (2012) who noticed an elevation in pro PO activity and super oxide anion (O2−) in feeding *P. monodon*, *C. dactylon* has antidiabetic and antibacterial activity (Kumar et al., 2008; Kaleeswaran et al., 2012).

As a sequence of the previous results of the present work, there is possible application of using Biogen, sodium alginate and herbs such as garlic and *C. dactylon* in aquaculture. The herbal medicines are also known to exhibit anti-microbial activity, facilitate growth, and maturation of cultured species; besides under intensive farming the anti-stress characteristics of herbs will be of immense use without posing any environmental hazard.

### References


