Effect of probiotics on immunological status of giant freshwater prawn
(*Macrobrachium rosenbergii* de Man)

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**ABSTRACT**

The present study was aimed to investigate hemocyte count in giant freshwater prawn (*Macrobrachium rosenbergii* de Man) after being fed with different supplements. Immunological status of an animal is reflected with the result of its hematological profile. Freshwater prawns are more prone to infections by several pathogens which influence the immunity of the affected individuals. The effect of two probiotics on hemocyte count was studied over a period of eight weeks. The level of hemocyte count decreased significantly in the blood of prawns administered with pathogenic organisms died after three weeks. The level of count of hemocyte showed significant increase in the blood of giant freshwater prawn treated with probiotics alone verses control and also in prawn administered with pathogenic organisms verses probiotics revealed that probiotic 1 was more effective than probiotic 2.

**Key words:** *Aeromonas hydrophila*, Bacteria, Hemocyte count, *Macrobrachium rosenbergii*, Probiotics, *Pseudomonas fluorescence*.

**INTRODUCTION**

Giant freshwater prawn is an economically important species because of its fast growth in tropical and subtropical regions. One of the major constraints limiting the prawn production all over the world is diseases. Generally, when compared to penaeid shrimp, *M. rosenbergii* is considered to be a moderately disease-resistant species. (Sahul Hameed *et al.*, 2004). Disease prevention has been a priority and shrimp immunology has become a prime area of research. As reported by Lightner (1992), the need to reduce the lethal and weakening effects of pathogens is stimulating a renewed interest in the defence mechanisms and the immune system of crustaceans. In shrimp, the most important role of the circulation hemocytes is the protection of animals against invading microorganisms by participating in recognition, phagocytosis and melanization (Tzou *et al.*, 2002). Despite the variety of shrimp responses, many of them originate from hemocyte. Shrimp hemocytes are involved in defence mechanisms such as phagocytosis, encapsulation, clot formation and melanization (Johansson *et al.*, 2000). Shrimp hemocytes are involved in defense processes such as phagocytosis, encapsulation, melanization and coagulation (Johansson *et al.* 2000). Thus, hemocyte number is sometimes used as an indicator of shrimp health status (Perazzolo *et al.* 2002).

Probiotics are the viable bacteria that beneficially influence the host by improving its intestinal microbial balance (Wang and Xu, 2006). These were found to be used in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Irianto and Austin, 2002). They are commonly defined as mono-or mixed cultures of live microbes that, when applied to animal or human, generate a beneficial effect on health of the host. These beneficial effects include disease treatment and prevention as well as improvement of digestion and absorption in the host (Nikfar *et al.*, 2010, Bansal *et al.*, 2011, Yesillik *et al.*, 2011). Fish larvae, shrimps and other invertebrates have immune systems that are less well developed than adult fish, and are dependent primarily on nonspecific immune responses for their resistance to infection. Probiotics improve resistance against infection by stimulation of host immunity (Havenaar and Huis in’t Veld, 1992). *Macrobrachium rosenbergii* were fed with *Lactobacillus sporogenes* as bio-encapsulated probiotic via *Artemia*, improved growth rate, feed efficiency ration of post-larvae and improved the immune system (Venkat *et al.* 2004). *Penaeus monodon* (PL-10) Post-larvae survival was higher when challenged with *V. harveyi*. Probiotic (*Bacillus S11*) provided cellular and humoral immune defence responses (Rengpipat *et al.* 2000). Probiotics were used in crustaceans (Anderson and Klontz, 1965) i.e. Freshwater prawns, *Macrobrachium rosenbergii* and shrimps (*Penaeus monodon*) (Ajitha *et al.*, 2004) for their health benefits (Chabrillon *et al.*, 2006). For the physiological state of an organism blood parameters are used as tool (Bansal *et al.*, 1979). The present investigation was conducted to determine the effects of probiotics on hemocyte count in giant freshwater prawn (*Macrobrachium rosenbergii de Man*) after being fed with different supplements.

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MATERIAL AND METHODS

The experiment in the present study was conducted in the Department of Zoology, CCS, Haryana Agricultural University, Hisar (India). In this experiment healthy individuals of prawns 3-4 months old were brought from the fish farms of this study to the laboratory and were acclimated at 25°C for one week in flat bottomed circular 30 L tubs. The tubs were filled with dechlorinated tap water which was daily removed and was also properly aerated. The prawns were fed a normal recommended commercial diet. Only the healthy prawns showing normal activities were selected for further experimentation.

Two commercial probiotics with different composition were used to control the infections caused by the pathogenic bacteria. **Probiotic 1** includes *-Lactobacillus casei, L. acidophilus, L. lactis, Streptococcus faecium, Bacillus coagulans, B. lincheniformis, saccharomyces cervisiae* and **Probiotic 2** includes *- Bacillus subtilis, B. licheniformis, B. polymyxa, B. megaterium, Aspergillus oryzae, Saccharomyces, Lactobacillus, Nitrosomonas, Nitrobacter, Pseudomonas putida.*

The following treatments were given to the prawns:

- **i) Control:** In this treatment, 200µl of physiological buffer saline (PBS) was injected between 2&3rd leg into the abdomen of each acclimated prawn.
- **ii) Control+**: *Aeromonas hydrophila*: Here, 200 µl bacterial suspensions with 5.2 x 10^11 cfu per ml of bacteria were injected between 2&3rd leg into the abdomen of prawns.
- **iii) Control+**: *Pseudomonas fluorescence*: Here, 200 µl bacterial suspensions with 5.2 x 10^11 cfu per ml of bacteria were injected between 2&3rd leg into the abdomen of prawns.
- **iv) Control+**: *A. hydrophila + P. fluorescense*: Here, 200 µl bacterial suspensions with 5.2 x 10^11 cfu per ml of bacteria were injected between 2&3rd leg into the abdomen of prawns.
- **v) Control + probiotic1**: Here, 0.1 gm probiotic1 with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **vi) Control + probiotic2**: Here, 0.1 gm probiotic 2 with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **vii) Control + A. hydrophila + probiotic1**: Here, 0.1 gm probiotic 2 along with 200 µl of bacterial suspension with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **viii) Control + P. fluorescense + probiotic1**: Here, 0.1 gm probiotic1 along with 200 µl of bacterial suspension with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **ix) Control + P. fluorescense + probiotic2**: Here, 0.1 gm probiotic2 along with 200 µl of bacterial suspension with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **x) Control + P. fluorescense + probiotic2**: Here, 0.1 gm probiotic1 along with 200 µl of bacterial suspension with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **xi) Control + (A. hydrophila + P. fluorescense) + probiotic1**: Here, 0.1 gm probiotic1 along with 200 µl of bacterial suspension with 5.2 x 10^11 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.
- **xii) Control + mixture of bacterium (A. hydrophila +P. fluorescense)+ probiotic2**: Here, 0.1 gm probiotic2 along with 200 µl of bacterial suspension with 5.2 x 10^10 cfu per ml of bacteria was injected between 2&3rd leg into the abdomen of prawns.

**Hematological Studies:** Blood parameters total leucocytes count (TLC) were determined with help of a haemocytometer and calculated from the equations given by Anderson and Klontz (1965).

**Collection of blood from giant freshwater prawn (Macrobrachium rosenbergii) under different treatments:** Blood samples of treated prawn were taken at weekly interval after initiation of treatments. Sampling was also done at the same time from control group. The sampling area was surface sterilized with 70% ethanol before injection. Haemolymph was withdrawn from the pericardial sinus into a 1 ml sterile syringe (21-gauge needle) containing anti-coagulant solution (.387% sodium citrate, EDTA). A drop of haemolymph suspension was placed on a haemocytometer and measured three times for the THC using a light microscope.

**Total hemocyte count:** Collection of hemolymph from Freshwater prawn (*Macrobrachium rosenbergii*) and calculation of hemocyte count.

1. The sampling area was surface sterilized with 70% ethanol before injection. Haemolymph was withdrawn from the pericardial sinus into a 1 ml sterile syringe (21-gauge needle) containing anti-coagulant solution (.387% sodium citrate, EDTA). Hemolymph was diluted 1:20 with WBC diluting fluids using WBC counting pipette. The mixture was shaken well to suspend the cells uniformly in the solution. Total hemocytes were determined using a haemacytometer and calculated as number of blood cells per mm^3^.
2. The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area of 1.0 sq mm therefore, 4 x 1.0 sq mm = a total of 4 sq mm). The volume counted is: area x depth = volume. Four sq mm x 0.1 mm = 0.4 cu mm.
3. The formula is as follows:

\[
\text{WBCs /cu mm} = \frac{\text{Average number of chambers} \times \text{WBCs counted x dilution 20}}{\text{Volume (.4)}}
\]

**Statistical analysis:** The obtained results were analyzed statistically using completely randomized design (CRD) to
RESULTS AND DISCUSSION

Level of total hemocyte count (per mm$^3$) in the blood of gaint freshwater prawn (M. rosenbergii): The hemocyte count level of normal diet fed prawns remained in the range of 1.880±0.008 to 2.160±0.016. However, in prawns inoculated with pathogenic $A$. hydrophila (T4) and $P$. fluorescence (T7) alone, the level of hemocyte decreased and remained in the range of 1.430±0.016 to 0.870±0.008 and 1.420±0.016 to 0.880±0.008 respectively. The range further decreased to 1.860±0.016 to 0.850±0.016 in prawns treated with both the bacterium together. Thus, the counts of hemocytes decreased steeply seemed to be due to increased production of $A$. hydrophila(T4), $P$. fluorescence (T7) and $A$. hydrophila + $P$. fluorescence (T10) as affects the immune system of prawns and decreased phagocytes. The decrease was more in $A$. hydrophila(T4), $P$. fluorescence (T7) and $A$. hydrophila + $P$. fluorescence (T10) indicated that $A$. hydrophila (T4) was more pathogenic than $P$. fluorescence (T7) then $A$. hydrophila + $P$. fluorescence(T10).

The hemocyte count remained in the range of 2.520±0.016 to 2.264±0.016 and 2.480±0.016 to 2.090±0.016 in prawn inoculated with $A$. hydrophila + probiotic1 (T5) and $P$. fluorescence + probiotic1 (T8), respectively. The level was in the range of 1.920±0.016 to 2.320±0.016 in prawn inoculated with $A$. hydrophila + $P$. fluorescence + probiotic2 (T11) and 1.960±0.016 to 2.520±0.016 in prawn inoculated $A$. hydrophila + $P$. fluorescence + probiotic1 (T12). However, the hemocyte count level was in the range of 2.400±0.163 to 2.560±0.016 and 2.320±0.016 to 2.090±0.016 in prawn inoculated with $A$. hydrophila + probiotic2 (T6) and $P$. fluorescence + probiotic 2 (T9), respectively, and in the range of 1.920±0.016 to 2.320±0.016 in prawn inoculated with $A$. hydrophila + $P$. fluorescence + probiotic2 (T11).

On the other hand, the prawn given the treatment of probiotics (P1 (T2) and P2 (T3)) showed maximal value of hemocyte count as compared to all other treatments including control. The level of hemocyte count was in the range of 3.000±0.016 to 3.315±0.457 in prawn administrated with P1 (T2) and in the range of 2.653±0.374 to 3.080±0.000 in prawn administrated with P2 (T3).

The normal counts fell in the range and degree of variation reported for Penaeus monodon by Van de Braak et al. (1996) and Owens and O’Neill (1997) (i.e. 5.0 x 10$^2$ ± 2 x 10$^2$ and 2.3 x 10$^4$ ± 1.4 x 10$^4$, respectively). Blood parameters are considered patho-physiological indicators of whole body and, therefore, are important in diagnosing the functional and structural status of fish invaded by the pathogens (Golovina, 1996).

The stress induced on the animals by exposing them to change in the water pH resulted in lowered immunological values. Lowered THC and PO activity were observed in Macrobrachium rosenbergii and some penaeids, after exposing the animals to various environmental stresses including hypoxia (Cheng et al., 2002; Perazzolo et al., 2002). Since precursors of PO system are stored within the hemocytes, decline in the hemocyte count may lead to a lowered serum PO activity. Comparisons of THC are difficult because of the wide variation amongst individual shrimp (Kallaya et al. 2005). Hematological parameters reflect the poor condition of fish more quickly than other commonly measured parameters. A number of haematological indices such as haemoglobin, red blood cells, white blood cells, packed cell volume and so on are used to assess the functional status of oxygen carrying capacity and defence system of the blood stream which enhances the immune system (Chinabut et al., 1995). However, very scanty work has been done on the blood parameters. Palikova et al. (2004) observed decrease in the level of blood in the common carp after exposure to Cyanobacteria extract. Ranzani-Paiva et al. (2004) showed that the decrease in erythrocytes count and haematocrit/PCV of Nile tilapia when inoculated with Mycobacterium marinum may lead to a tendency to anaemia.

Irianto and Austin (2002) observed an increase in erythrocyte count in fish, fed on probiotic bacteria than control group. Rajesh et al. (2008) reported that the probiotics used in carps increased the level of blood parameters as a result of hemopiotic stimulation when fed on probiotic bacteria. Irianto and Austin (2002) used dead probiotic cells to control disease and observed higher number of leucocytes, erythrocytes and macrophages in rainbow trout, (Oncorhynchus mykiss). Rengpipat et al. (2000) and Siwicki et al. (2003) found disease resistance or protection tiger shrimp (P. monodon) when Bacillus sp. was used as probiotics and also reported increase in the level of their selected hematological parameters in the blood e.g. red blood cell count, hematocrit, haemoglobin and various leukocyte counts. Selvaraj et al. (2005) reported increase in the total leukocyte counts, and an increase in proportion of neutrophils and monocytes in C. carpio when the fish were fed with fungus (Saccharomyces cerevisiae).

The present work revealed that P1 gives better results in increasing the hemocyte count of prawns as compared to P2 inoculated with pathogenic $A$. hydrophila (T4) and $P$. fluorescence (T7) alone and $A$. hydrophila + $P$. fluorescence (T10) together.

The variety of shrimp defense responses, many of them originate from hemocytes. One approach to overcome disease problems in shrimp aquaculture has been the development of feed additives called immunostimulants which may increase shrimp defence against potential pathogens (Smith et al. 2003).

In conclusion, the results of present study revealed that probiotic had a positive effect on hemocyte count i.e. (Table 1). This clearly indicated that there was increased in
Table 1: Effect of probiotics on the total hemocytes of gaint freshwater prawn (M. rosenbergii)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total hemocytes (per mm4) in different weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Normal diet (T1)</td>
<td>1.880±0.008</td>
</tr>
<tr>
<td>Probiotic 1(T2)</td>
<td>3.000±0.016</td>
</tr>
<tr>
<td>Probiotic 2(T3)</td>
<td>2.653±0.374</td>
</tr>
<tr>
<td>A. hydrophila(T4)</td>
<td>1.430±0.016</td>
</tr>
<tr>
<td>A. hydrophila + Probiotic 1 (T5)</td>
<td>2.520±0.016</td>
</tr>
<tr>
<td>A. hydrophila + Probiotic 2 (T6)</td>
<td>2.400±0.163</td>
</tr>
<tr>
<td>P. fluorescense (T7)</td>
<td>1.420±0.016</td>
</tr>
<tr>
<td>P. fluorescense + Probiotic 1 (T8)</td>
<td>2.480±0.016</td>
</tr>
<tr>
<td>P. fluorescense + Probiotic2 (T9)</td>
<td>2.320±0.016</td>
</tr>
<tr>
<td>A. hydrophila +</td>
<td>1.860±0.016</td>
</tr>
<tr>
<td>P. fluorescense(T10)</td>
<td></td>
</tr>
<tr>
<td>A. hydrophila + P. fluorescense + Probiotic 2(T11)</td>
<td>1.920±0.016</td>
</tr>
<tr>
<td>A. hydrophila + P. fluorescense + Probiotic 1(T12)</td>
<td>1.960±0.016</td>
</tr>
<tr>
<td>CD value</td>
<td>0.1701</td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td></td>
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</tbody>
</table>

Values are as Mean±SD. N=27 (9 prawns X 3 replications)

(-) prawns died after 3 weeks
the value of hemocytes of prawns. Thus, the new generation of alternative measures for the prevention of bacterial diseases in prawn which should be used for maintaining the prawn health. Probiotic 1 should be preferred over Probiotic 2 for preventing the out break of diseases in prawns.

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