Efficacy of two commercial and indigenous probiotics, Bacillus subtilis and Bacillus licheniformis on growth performance, immuno-physiology and resistance response of juvenile white shrimp (Litopenaeus vannamei)

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A R T I C L E  I N F O

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Probiotic
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A B S T R A C T

This study aimed to assess efficacy of commercial and indigenous sources of Bacillus subtilis and Bacillus licheniformis as probiotic dietary supplementation on the growth performance, immuno-physiological variables and resistance response to some stressors in white leg shrimp (Litopenaeus vannamei) weighing 50 ± 6.54 mg at 28 °C for 60 days. Shrimps were fed with four different diets: control (without probiotic); diets supplemented with commercial probiotics(CP) each at 1.5 × 10^6 CFU/g; commercial + indigenous probiotics(CIP) each at 1.5 × 10^6 CFU/g (four strains each at 7.5 × 10^5 CFU/g) and indigenous probiotics (IP) each at 1.5 × 10^6 CFU/g. Growth and feeding parameters including final weight (FW), specific growth rate (SGR) and feed conversion ratio (FCR) were significantly influenced by all probiotics compared to control one (P < .05), with a better growth performance and survival observed in CP group than other treatments. Glucose and cortisol values in all treated shrimps were lower than control one but was only significantly different for CP group (P < .05). Values of albumin, total protein, and lysozyme in CP group was mostly insignificantly higher than other two treated groups, except for lysozyme value that was significantly different (P < .05). In addition, total haemocyte count (THC), large granular cells (LGC), semi-granular cells (SGC) and hyaline cells (HC) significantly increased in CP group compared to other treatments and control but were only significantly different with control one. However, the values of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were significantly lower in all treatment groups compared with control shrimps, except for ALP. Shrimps fed with all types of probiotics showed a higher resistance to environmental stressors including low and high salinities, formalin, chlorine, high and low water temperatures and ammonia but such resistance was more prominent in CP group. The load of Bacillus bacteria in gut of CP treatment was significantly higher than other two groups and control, and that of CIP group was also significantly higher than IP group (P < .05). These data show that there are some differences in efficacy and potency between the commercial and indigenous probiotic sources in growth and health condition of L. vannamei, and thus requires further attention and research works.

1. Introduction

Shrimp farming is now considered as a significant economic food production and one of the most valuable activities in aquaculture industry. The shrimp farming has influenced as a progressively important source of protein available for human consumption over the past five years (Lakshmi et al., 2013). As the fast growth of shrimp culture in tropical countries, problems related to diseases and deterioration of environmental conditions have been seriously considered (Zokaifar et al., 2012; Interaminense et al., 2018). Outbreaks of infectious
diseases is still one of the primary and critical limiting factors to the growth of shrimp farming, thus prevention criteria compared to disease treatment is of high priority (Lakshmi et al., 2013). The vast usage of chemical substances, especially antimicrobial agents in shrimp farming has left enormous negative impact on the biosphere; further the abuse of antimicrobials can end in the development of resistant bacterial pathogens. Traditionally, the control and prevention of bacterial diseases in penaeid hatcheries has depended on the use of chemical compounds; nonetheless, based on recent studies, other alternatives such as probiotic therapy have been examined to control the infectious diseases not only in shrimp aquaculture but also in other farmed aquatic species (Kumar et al., 2016).

*Bacillus* spp. are Gram-positive and rod-shaped bacteria with ability to produce a robust spore and grow efficiently in conditions with very low-cost carbon and nitrogen sources (Buriana et al., 2014). Most *Bacillus* species are not harmful to mammals, including humans, and are introduced as producers of a various amounts of secondary metabolites containing antibiotics, beneficial chemicals and enzymes. The advantage features of *Bacillus* have made them good candidates as probiotics in shrimp diets (Zokaeifar et al., 2012, 2014). These Gram positive spore-forming bacteria have been used to enhance the growth, health status, disease resistance as well as water quality parameters of some cultured species of fish and shellfish including shrimp with mostly promising results (Zaei-Nejad et al., 2006; Balcazar et al., 2007; Li et al., 2007; Keysami et al., 2007; Tseng et al., 2009; Shen et al., 2010; Liu et al., 2010; Luis-Villaseñor et al., 2011; Nimrat et al., 2012; Seenivasan et al., 2012; Zokaeifar et al., 2012; Zokaeifar et al., 2014; Kumar et al., 2013; Kumar et al., 2016; Interaminenese et al., 2018). Use of commercial *Bacillus subtilis* and *Bacillus licheniformis* as probiotics either in a single form or in the combination manner has raised a promising hope for aquaculture species including shrimp. However, there is now a question says; is there any difference between the efficacy and potancy of commercial and indigenous probiotic products on growth and health status of aquatic animals? The hypothesis is that an indigenous probiotic may provide a better efficacy on growth and health condition of the target animal than commercial one as it is less manipulated under laboratory condition or production process. In contrast, a commercial probiotic could be more effective than the indigenous one because the commercial strains are selective in their characteristics e.g. levels of enzyme products, survival in the gastrointestinal environment, ability to withstand low pH and high concentrations of bile acids and other desirable characteristics. As there is no data available therefore, this study was aimed to investigate the influence of commercial and indigenous probiotic sources of these two mentioned *Bacillus* species on the growth performance, immunophysiological variables, and stress tolerance in white shrimp, *L. vannamei*.

2. Materials and methods

2.1. Shrimp

Shrimps (PL1+) were obtained from a shrimp hatchery centre in Gomishan shrimp farming region, North Iran, and were acclimatized in two fiberglass tanks for 15 days prior to the experiment. Shrimps weighing 50 ± 6.54 mg were then randomly stocked in 50 L tanks of filtered (cheesecloth filter) sea water at a density of 50 shrimps/tank with a constant water temperature of 28 °C. Moults, waste products and dead shrimps were removed prior to each feeding time, and water exchange was performed at 10% a day during the experiment (Olmos et al., 2011).

2.2. Probiotics

Commercial probiotics, *Bacillus subtilis* and *B. licheniformis* spores (Protexin Probiotics International Ltd., Somerset, UK) were activated for eight h before being grown in tryptic soy agar (TSA) at 30 °C for 24 h. These probiotics are normally used in both poultry and swine. The indigenous probiotics, *B. licheniformis* (BL-HH) and *B. subtilis*(BS-HH) previously isolated from the gut of *Huso huso* fingerlings (aquatic microbiology laboratory, department of aquatic animal health, faculty of veterinary medicine, University of Tehran) were grown in TSA at 30 °C for 24 h. The final concentration of each bacterial suspension of either indigenous or commercial probiotics was adjusted to desirable concentrations for spreading on the feed by spectrophotometry (610 nm) (Biochrom Libra S22) followed by viable counting on TSA (Zokaeifar et al., 2012).

2.3. Feed preparation

Commercial feed (Haurash, Bushehr, Iran) with composition of protein 38%, lipid 9% and energy 4523 cal/g was used as the basal diet. The suspensions of probiotics in dissolved cooked starch (40 g/L) were sprayed on the feed (80 mL probiotic suspension/kg feed) before being mixed thoroughly. The concentration of probiotics in feed was then determined using spread-plate count by making a 10-fold serial dilution of 1 g feed in phosphate-buffered saline (PBS; pH 7.2) before spread plate counting (100 μL of each dilution per plate in three replicates). Mannitol-egg yolk-polymyxin (MYP) agar (Difco) was used to estimate probiotic concentration in feed (CFU/g) (Zokaeifar et al., 2012). The control shrimps were fed with the basal diet spread with starch but without the probiotics.

2.4. Experimental design and growth condition

Shrimps were fed with four diets in triplicate each 50 shrimps: Control diet without probiotic (C); commercial probiotics (CP) (*B. licheniformis*, *B. subtilis* each at 1.5 × 10⁶ CFU/g feed), indigenous probiotic (IP) (*B. licheniformis*, *B. subtilis* each at 1.5 × 10⁶ CFU/g feed) and commercial + indigenous probiotics (CIP) (four strains each at 7.5 × 10⁵ CFU/g feed (1:1 proportion). Shrimps were fed at 7% body weight three times a day (at 06:00, 14:00 and 22:00 h) for 60 days. Feed intake, mortality and water quality parameters were recorded daily (Pazir et al., 2008). At the end of the experiment survival rate, immunological variables, growth and feeding parameters as well as density of *Bacillus* bacteria in the shrimp gut were evaluated as described below.

2.5. Growth factors and survival

Growth factors including weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), thermal growth coefficient (TGC), relative gain rate (RGR) and daily growth coefficient (DGC) were calculated using below equations. Survival rate (SR) was recorded by counting daily mortality in each tank and was calculated for each treatment by following the equations.

\[
\text{RGR} = \frac{100}{\text{T}} \times \frac{\text{WG}}{\text{WG}} = \frac{100}{\text{T}} \times \frac{\text{WG}}{\text{WG}}
\]

\[
\text{WG} = 100 \times (\text{W}_0 - \text{W}_f)/\text{W}_0
\]

\[
\text{SR} = 100 \times (\text{W}_0 - \text{W}_f)/\text{W}_0
\]

\[
\text{TGC} = \frac{W_t - W_0}{T} \times (t_2 - t_1)
\]

\[
\text{DGC} = \frac{W_t - W_0}{(t_2 - t_1)}
\]

\[
\text{FCR} = \frac{\text{total feed intake (g)}}{\text{total wet weight gain (g)}}
\]

\[
\text{WG} = \frac{100 \times (\text{W}_0 - \text{W}_f)/\text{W}_0}{\text{T}}
\]

\[
\text{FCR} = \frac{\text{total feed intake (g)}}{\text{total wet weight gain (g)}}
\]

\[
\text{SR} = 100 \times (\text{W}_0 - \text{W}_f)/\text{W}_0
\]

\[
\text{TGC} = \frac{W_t - W_0}{T} \times (t_2 - t_1)
\]

\[
\text{DGC} = \frac{W_t - W_0}{(t_2 - t_1)}
\]

\[
\text{FCR} = \frac{\text{total feed intake (g)}}{\text{total wet weight gain (g)}}
\]
turbidimetric assay described by Chakrabarti et al. (2014) using 2006). Colony forming unit (CFU) per mL were then calculated after in sterile PBS prior to spreading on TSA at 37 °C for 72 h (Mahious et al., probiotics (four strains each at 7.5 × 10⁵ CFU/g); IP: diet supplemented with indigenous probiotics each at 1.5 × 10⁶ CFU/g.

10 min each. A total of 200 cells were counted on each slide and total di—different haemocyte cells (DHCs) were counted by a haemocytometer slide under a

and hen chicken lysozyme (Sigma). Total haemocyte cells (THCs) were counted by a haemocytometer slide under a

method (Doumas et al., 1981). Lysozyme activity was estimated by a

determined using Bromo cresol green method using Pars Azmuns kits (Iran) in an auto—analyzer (Eppendorf). Serum total protein was examined by Biuret method (Doumas et al., 1981). Lysozyme activity was estimated by a turbidimetric assay described by Chakrabarti et al. (2014) using Micrococcus lysodeikticus and hen chicken lysozyme (Sigma). Total haemolymph cells (THCs) were counted by a haemocytometer slide under a light microscope compound (× 400). One drop of the haemolymph previously mixed with the anticoagulant was smeared onto a microscope glass slide. The smears were air dried, fixed in 70% methanol for 10 min before being stained with May-Grunwald and Giemsa stains for 10 min before being stained with May-Grunwald and Giemsa stains for

9 mmol EDTA, pH 7.0) and centrifuged at 1400 × g for 10 min before being stained with May-Grunwald and Giemsa stains for

extended in 70% methanol for

dispensed into microtubes containing 0.4 mL of Alsever’s anticoagulant solution (115 mmol glucose, 336 mmol NaCl, 27 mmol citrate sodium, 9 mmol EDTA, pH 7.0) and centrifuged at 1400 × g for 10 min before being stained with May-Grunwald and Giemsa stains for

and centrifuged at 1000 × g for 10 min before being stained with May-Grunwald and Giemsa stains for

total number of shrimp stocked) 100 (Kumar et al., 2013)

2.6. Immunological assays

Haemolymph samples of six shrimps in each tank (18 shrimps per trail) were randomly obtained and mixed as a pool sample (Kongnum and Hongpattarakere, 2010). The haemolymphs were obtained from the pericardial cavity of individual shrimp (Hai and Fotedar, 2009) and dispensed into microtubes containing 0.4 mL of Alsever’s anticoagulant solution (115 mmol glucose, 336 mmol NaCl, 27 mmol citrate sodium, 9 mmol EDTA, pH 7.0) and centrifuged at 1400 × g for 10 min (Prodoscimo et al., 2007). The collected haemolymph samples were kept at 4 °C before being tested. Glucose content was measured by photo—

growth factors measured in treated groups were significantly higher in the IP group (P > .05). In addition, except FCR and SGR in which

SR (%) = (total number of shrimps survived /total number of shrimp stocked) 100 (Kumar et al., 2013)

2.8. Enzyme activities

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in haemolymph samples were evaluated by method described by Thomas (1998), and alkaline phosphatase (ALP) was estimated according to Bergmeyer et al. (1986).

2.9. Stress test

At the end of the feeding trial, five shrimps from each replicate (15 per trail) were randomly exposed to stress tests including low and high salinities (5 and 100 ppt), formalin at 5 mg/L, chlorine at 200 mg/L, water temperature at 8 °C and 20 °C and ammonia at 5 mg/L. The challenge tests were run till all the shrimps died and time (min) of tolerance was calculated (Jafaryan and Soltani, 2012).

tolerance was calculated (Jafaryan and Soltani, 2012).

2.10. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA). To compare the data, Duncan procedure was performed at 5% level.

3. Results

3.1. Growth performance

Results of growth indices and survival are shown in Table 1. All growth factors measured in treated groups were significantly improved compared to control one (P < .05), except for SGR and FCR in which were insignificant between IP group and control shrimp (P > .05). Also, the growth factors in CP and CIP groups were significantly improved compared to IP group (P < .05), except for FCR that was significantly higher in the IP group (P > .05). In addition, except FCR these growth factors in CP group were significantly higher than both IP and CIP groups (P < .05). The highest and the lowest survival rates (SR) were observed in CP (83.5 ± 2.45%) and control (72 ± 3.25%) groups, respectively (P < .05). Also, SR in CP was higher than both CIP (80 ± 3%) and IP (77 ± 2.5%) groups but was only significantly different with IP group (P < .05).

Table 1

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Treatment</th>
<th>CP</th>
<th>CIP</th>
<th>IP</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW (mg)</td>
<td></td>
<td>1101.12 ± 48.13 ± 69.94</td>
<td>982.71 ± 69.94</td>
<td>888.06 ± 57.72</td>
<td>811.62 ± 66.69</td>
</tr>
<tr>
<td>FL (mm)</td>
<td></td>
<td>58.46 ± 6.36</td>
<td>54.15 ± 5.02</td>
<td>52.30 ± 5.49</td>
<td>50.28 ± 5.57</td>
</tr>
<tr>
<td>SGR (%)</td>
<td></td>
<td>4.78 ± 0.48</td>
<td>4.63 ± 0.39</td>
<td>4.47 ± 0.44</td>
<td>4.36 ± 0.32</td>
</tr>
<tr>
<td>TGC (%)</td>
<td></td>
<td>0.39 ± 0.06</td>
<td>0.37 ± 0.05</td>
<td>0.35 ± 0.05</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>DGC (%)</td>
<td></td>
<td>1.10 ± 0.18</td>
<td>1.04 ± 0.14</td>
<td>0.99 ± 0.15</td>
<td>0.59 ± 0.11</td>
</tr>
<tr>
<td>RGR (%)</td>
<td></td>
<td>2252.8 ± 143.9</td>
<td>2001.9 ± 176.8</td>
<td>1797.6 ± 150.7</td>
<td>1634.3 ± 156.2</td>
</tr>
<tr>
<td>FCR (%)</td>
<td></td>
<td>1.78 ± 0.56</td>
<td>1.93 ± 0.48</td>
<td>2.17 ± 0.62</td>
<td>2.29 ± 0.51</td>
</tr>
<tr>
<td>SR (%)</td>
<td></td>
<td>83.5 ± 2.45</td>
<td>80 ± 3</td>
<td>77 ± 2.5</td>
<td>72 ± 3.25</td>
</tr>
</tbody>
</table>

FW: final weight; FL: final length; SGR: specific growth rate; TGC: thermal growth coefficient; DGC: daily growth coefficient; RGR: relative gain rate; FCR: Feed conversion ratio; SR: survival rate. Values (means ± SE) with different superscripts on the same row showing significant difference (P < .05).
The results of stress tests are shown in Table 3. The stress resistance in all treated shrimps were significantly higher than control shrimps (P < .05). Also, shrimps fed with CP probiotics demonstrated significantly a better resistance to the stressors than shrimps fed IP probiotics (P < .05). ALP, AST and ALT in the experimental groups were lower than control but were mostly significantly different for ALT and AST (P < .05) (Table 2).

3.3. Stress resistance

The results of stress tests are shown in Table 3. The stress resistance in all treated shrimps were significantly higher than control shrimps (P < .05). Also, shrimps fed with CP probiotics demonstrated significantly a better resistance to the stressors than shrimps fed IP probiotics (P < .05). Also, CP group showed significantly a higher resistance response to formalin, low salinity and low temperature than shrimps fed with mixture of both probiotic products (P < .05). Stress resistance in shrimps fed with CIP probiotics were also improved compared to IP group but were significant only for formalin and basic pH (P < .05).

3.4. Total count of Bacillus spp.

Population densities of Bacillus bacteria counted in the digestive tract of shrimps treated with probiotics are given in Fig. 1. The concentration of Bacillus bacteria in all treated groups were significantly higher than control shrimps (P < .05). Also, this bacterial population in CP group was significantly higher than other two groups, and that of CIP group was significantly higher than IP one (P < .05).

4. Discussion

Nowadays, manipulation and application of probiotics including Bacillus bacteria have been reported as a worthy practice for aquaculture not only to improve growth condition but also enhance immune status with a consequence in increasing in disease resistance. So far, few studies have been carried out to assess the efficacy of Bacillus bacteria as potential probiotics in shrimp aquaculture. In most studies, a promising effect was seen when Bacillus bacteria were used either as a bath or orally in feed (Zokaeifar et al., 2014; Liu et al., 2010). However, there is no data regarding the comparison effect between the commercial and indigenous sources of Bacillus spp.

Results of this study showed oral administration of commercial B. *subtilis* have a promising effect on the growth and disease resistance of Litopenaeus vannamei shrimp. This study also indicated the use of probiotics to improve growth and disease resistance in Litopenaeus vannamei shrimp and it also presents a source of probiotics that can be used in shrimp aquaculture.

Table 2

<table>
<thead>
<tr>
<th>Immuno-physiological value</th>
<th>CP</th>
<th>CIP</th>
<th>IP</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>486.6 ± 29014^b</td>
<td>650.0 ± 23.09^ab</td>
<td>716.60 ± 31.79^a</td>
<td>746.00 ± 17.77^a</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>1.19 ± 0.08^a</td>
<td>1.15 ± 0.01^a</td>
<td>1.14 ± 0.02^a</td>
<td>1.12 ± 0.01^a</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.79 ± 0.01^a</td>
<td>5.69 ± 0.08^b</td>
<td>5.48 ± 0.01^b</td>
<td>5.11 ± 0.05^b</td>
</tr>
<tr>
<td>Cortisol (mg/g)</td>
<td>7.65 ± 0.25^b</td>
<td>7.85 ± 0.43^b</td>
<td>8.50 ± 0.23^b</td>
<td>10.67 ± 0.51^b</td>
</tr>
<tr>
<td>Lysozyme (U/ml/min)</td>
<td>79.00 ± 4.61^a</td>
<td>69.00 ± 2.88^a</td>
<td>64.00 ± 2.30^a</td>
<td>34.00 ± 1.00^a</td>
</tr>
<tr>
<td>Total haemocyte (x 10^6 cells/ml)</td>
<td>13.4 ± 0.13^a</td>
<td>12.6 ± 0.23^b</td>
<td>12.00 ± 0.10^b</td>
<td>10.7 ± 0.20^b</td>
</tr>
<tr>
<td>Large granular cell (x 10^6 cells/ml)</td>
<td>2.40 ± 0.11^a</td>
<td>2.05 ± 0.04^b</td>
<td>1.60 ± 0.02^b</td>
<td>1.20 ± 0.05^b</td>
</tr>
<tr>
<td>Hyaline cell (x 10^6 cells/ml)</td>
<td>3.45 ± 0.05^a</td>
<td>3.25 ± 0.08^ab</td>
<td>3.20 ± 0.03^ab</td>
<td>2.90 ± 0.02^ab</td>
</tr>
<tr>
<td>ALT (IUL–1)</td>
<td>7.60 ± 0.17^a</td>
<td>7.30 ± 0.13^a</td>
<td>7.20 ± 0.11^a</td>
<td>6.60 ± 0.15^a</td>
</tr>
<tr>
<td>AST (IUL–1)</td>
<td>125.00 ± 8.66^a</td>
<td>136.00 ± 9.23^b</td>
<td>147.50 ± 3.17^bc</td>
<td>651.00 ± 4.04^c</td>
</tr>
<tr>
<td>ALP (IUL–1)</td>
<td>1045.00 ± 60.62^c</td>
<td>1185.00 ± 63.50^b</td>
<td>1340.00 ± 86.60^b</td>
<td>1560.00 ± 80.82^c</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Stressor</th>
<th>CP</th>
<th>CIP</th>
<th>IP</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine (200 mg/L)</td>
<td>57.48 ± 4.28^a</td>
<td>52.21 ± 2.45^ab</td>
<td>44.05 ± 1.42^bc</td>
<td>38.30 ± 4.22^c</td>
</tr>
<tr>
<td>Formalin (5 mg/L)</td>
<td>31.36 ± 1.18^a</td>
<td>27.92 ± 1.33^a</td>
<td>24.39 ± 1.52^b</td>
<td>18.86 ± 1.63^c</td>
</tr>
<tr>
<td>Salinity (5 g/L)</td>
<td>198.14 ± 16.48^b</td>
<td>154.67 ± 7.93^b</td>
<td>143.81 ± 12.70^b</td>
<td>96.50 ± 9.85^c</td>
</tr>
<tr>
<td>Salinity (100 g/L)</td>
<td>142.70 ± 10.71^a</td>
<td>120.02 ± 4.66^ab</td>
<td>108.05 ± 4.18^ab</td>
<td>72.57 ± 11.48^c</td>
</tr>
<tr>
<td>Temperature (8 °C)</td>
<td>19.95 ± 1.95^a</td>
<td>17.05 ± 1.15^a</td>
<td>15.28 ± 0.67^a</td>
<td>10.78 ± 1.49^a</td>
</tr>
<tr>
<td>Temperature (40 °C)</td>
<td>5.47 ± 0.76^a</td>
<td>5.26 ± 0.37^ab</td>
<td>3.22 ± 0.63^bc</td>
<td>2.03 ± 0.43^c</td>
</tr>
</tbody>
</table>

Values (means ± SE, n = 3 pool samples) with different superscripts on the same row show significant difference (P < .05).
subtilis and B. lichenformis gave a superior growth in L. vannamei compared to indigenous strains of these Bacillus species. Even when the commercial strains were mixed with the indigenous ones equally at 50% concentration each, still a better growth performance was observed than indigenous group alone. The reason why the commercial strains of these Bacillus bacteria provided a better growth performance is unknown. However, the genetic of bacterial strain, original of the host from which the bacteria were isolated and the number of in vitro passages can affect the physiology a particular bacterial strain as a probiotic. Such changes in bacterial physiology obviously could make a change in efficacy and potency when it is used as a potential probiotic. The commercial probiotics used in this present study were a Protexin product (UK) with unknown origin host, while the indigenous bacteria were recovered from the shrimp intestine. Therefore, difference in the probiotic sources may affect their efficacies in the target animal.

At the end of experiment, shrimps fed with commercial probiotics gave 83.5 ± 2.4% survival, while the survival rates in commercial + indigenous and indigenous probiotics were 80 ± 3 and 77 ± 2.5%, respectively, again showing a superiority of the commercial probiotics compared to indigenous and even to the mixture one. This was supported by the counting of the population density of immunocompetent cells which demonstrated a higher number of defensive cells i.e. THC, HC, HGC and SGC in shrimps fed with the commercial probiotics than other treatments, especially were significantly higher than indigenous group. These immunocompetent cells of hemolymph are responsible for various immune-functions including phagocytosis, degranulation, encapsulation, node formation and prophenol oxidase cascade in Penaeidae including L. vannamei species (Sritunyalucksana and Söderhäll, 2000). Therefore, significantly a higher count of these immune defense cells in shrimps fed commercial probiotics is reflecting a better immunostimulatory effect by the commercial products than indigenous ones. In a study by Zokaeifar et al. (2012) a better growth performance and survival was seen in L. vannamei fed with B. subtilis previously recovered from the fermented pickles. These authors demonstrated an up-regulation of some immune related genes such as prophenol oxidase and serine protein in the shrimps fed with B. subtilis compared with control group. Therefore, a higher haemocyte cells in the shrimps fed the probiotics can lead to an enhancement in the animal immune responses. Similar findings were seen by Chiu et al. (2007) who reported an upregulation of prophenol oxidase and disease resistance in shrimps fed with L. plantarum-supplemented diet.

AST, ALP and AST are enzymes responsible for some biochemical reactions of metabolism that interconvert amino acids with other metabolites. In vertebrates, AST exists in two isoforms forms; mitochondrial and cytoplasmic forms with the highest level in tissues of heart, liver, muscle, and kidney, respectively. The increase of mitochondrial AST in blood is highly suggestive of tissue damage e.g. chronic liver diseases. ALT in tissues of liver, kidney, heart and muscle catalyzes the transamination reaction. Enzyme of ALP in the cells ducts of the liver can be found on the mucosal epithelium of the small intestine, kidneys, bone and liver tissues. It plays an important role in lipid transposition in small intestines and calcification of bones. Hepatopancreatic disorders cause an elevation in these enzymes. For example a rise in ALT is associated with reduced insulin response, reduced glucose tolerance, and increased free fatty acids and triglycerides. In studies by Anand et al. (2015, 2017) application of biofloc and periphyton reduced levels of AST and ALT, while enhanced growth and immune responses in P. monodon compared to control group. Therefore, measuring of these enzymes is one of well-known indicators to assess the hepatopancreas functions (Van de Braak et al., 2002). In this study, a lower value of these enzymes was measured in the shrimps fed with commercial Bacillus. We did not measure digestive enzymes such as lipase, protease and amylase which can be increased by Bacillus spp. in shrimp (Wang, 2007; Yu et al., 2008; Ziaei-Nejad et al., 2006), but a lower level of AST, ALP and ALT measured in shrimps fed commercial probiotics indicates a better function of hepatopancreas in this group compared to other treatments and control one. This could be in part due to a better physiological performance by commercial probiotics strains in terms of improving of digestive enzymes giving a better growth index as we found a better growth in commercial probiotics treatment than other treatments and control. In addition, this is supported by lower levels of glucose and cortisol measured in shrimps fed with the commercial probiotics compared to other treatments and control, as lower levels of these factors mean the animal is in a reduced stress condition (Liu et al., 2010; Mohapatra et al., 2013).

Hemocytes are the sources of some enzymes and proteins with immunological functions such as lysozyme and albumin. Recent works by Interaminense et al. (2018) showed that oral use of B. subtilis significantly improved L. vannamei growth and developed in shrimp hepatopancreas and intestine. However, only feed supplemented with mixture of Shewanella algae and B. subtilis could control Vibrio load in the shrimp body. In our study, similar to higher counts of hemocytes, shrimps fed with commercial probiotics also demonstrated higher levels of lysozyme activity, total protein and albumin than other two treatments and control one. Therefore, an increase in these values again is a supportive for the superiority of the efficacy and potency of commercial Bacillus species to the indigenous ones.

Najmi et al. (2018) assessed the efficacy of a commercial Lactobacillus probiotic named Protexin containing seven species: Lactobacillus plantarum, L. delbrueckii, L. acidophilus, Bifidobacterium bifidum, Streptococcus salivarius, Enterococcus faecium at 2 × 10^7 cells/g on growth and resistance response of L. vannamei larvae against salinity (10 and 40 ppt) and formalin (100 ppm) under unknown water quality. Resistance response (survival rate) in shrimps fed the probiotics significantly enhanced compared to control ones. In our study, when treated L. vannamei were subjected to various chemicals and environmental stressors, an increase stress resistance was seen in all Bacillus treated shrimps compared to control groups. However, shrimps fed with commercial probiotics demonstrated a better resistance than indigenous group. Such resistance is obviously due to better immunological functions of the animal, as higher levels in immune defensive cells, lysozyne activity, total protein as well all a lower cortisol level were measured in the commercial group compared to indigenous one.

Population density of Bacillus bacteria of both commercial and
indigenous sources counted in digestive tract of treated shrimps were significantly higher than the control group. Also, shrimps fed with commercial source of probiotics significantly revealed a higher concentration of the <i>Bacillus</i> bacteria in their guts than shrimps administered indigenous <i>Bacillus</i> species. <i>Bacillus</i> bacteria could multiply in the digestive tract of marine organisms (Interaminense et al., 2018) acting almost as oral vaccines. Various behavioral factors including adhesion properties, bacterial attachment site, stress factors, diet and environmental condition e.g. hardness, dissolved oxygen, temperature, pH and osmotic pressure have been demonstrated by the probiotic bacteria; and environmental microflora can affect bacterial colonization, proliferation and function of the probiotics in the animal digestive tract (Kumar et al., 2016). Vieira et al. (2006) also reported that in aquatic organisms, the largest portion of gut bacterial microbiota dedicated to Gram-negative, and, genera of <i>Vibrio</i>, <i>Pseudomonas</i>, and <i>Aeromonas</i> are predominant; and bacterial microbiota could be moderated by the addition of Gram-positive bacteria in the diet or in the culture water (Kongnum and Hongpattarakere, 2010).

An efficient methodology for binding probiotics to the feed granules needs to be developed. Mixing spore-producing bacteria such as <i>Bacillus</i> bacteria with feed ingredients during the manufacturing process may be a way of saving effort as preparation of feeding probiotics requires extra time and effort. Although, the heat tolerance levels vary among spore bacteria and related studies are further required, the endospore forming <i>Bacillus</i> can be considered as good candidates to over come such obstacle.

In conclusion, results of this work clearly show that application of two <i>Bacillus</i> bacteria, <i>B. subtilis</i> and <i>B. licheniformis</i> as the commercial products provided a superior efficacy and potency on growth and immunophysiology of <i>L. vannamei</i> compared with the same species of these <i>Bacillus</i> bacteria but with an indigenous source. Also, administration of a mixture of commercial and indigenous sources of these <i>Bacillus</i> species in shrimps provided better growth condition and immune responses than the indigenous <i>Bacillus</i> alone. The reason why such differences were seen is unknown and warrants further works. However, it may in part be due to the genetic differences of bacterial strains or differences in the process of probiotic preparation and production as well as the probiotic substances. Therefore, in aquaculture practice it is important to carefully assess the product source of probiotics before making a decision of their utilizations.

Authors contributions

The in vivo works were carried out by Dariuosh Abdollahi-Arpanahi and Mahsa Naderi Samani; Elahe Soltani helped microbiological works and preparation of revised version of the manuscript, Hojatollah Jafaryan and Mehdi Soltani conceived the project, designed and supervised the experiment and interpretation, Mehdi Soltani prepared the manuscript and Angel Isidro Campa-Górdova helped in data analysis.

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Authors declare that they have no competing interest. The research project was carried out according to the guidelines of the Ethics Committee for Research on Animals at the University of Tehran.

Conflict of interest

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