Synbiotic effect of *Bacillus mycoides* and organic selenium on immunity and growth of marron, *Cherax cainii* (Austin, 2002)

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Abstract

The present feeding trial examined the effect of synbiotic use of *Bacillus mycoides* and organic selenium (OS) as Sel-Plex on marron immunity, growth and survival. The marron were cultured in recirculated tanks and fed test diets consisting of a basal diet; basal diet supplemented with *B. mycoides* (10⁸ CFU g⁻¹ of feed); basal diet supplemented with OS (Sel-Plex) (0.2 g kg⁻¹ of feed) and basal diet supplemented with synbiotic (*B. mycoides* at 10⁸ CFU g⁻¹ and OS 0.2 g kg⁻¹ feed) diet, in triplicate. The effect of the prebiotic OS (Sel-Plex) on the growth rate of *B. mycoides* was also studied in vitro. The results suggested that synbiotic use of *B. mycoides* and OS significantly improved some immune parameters of marron, particularly the glutathione peroxidase, and to some extent total haemocyte counts. However, the synbiotic feed did not synergistically improve marron growth, in fact the use of *B. mycoides*-supplemented diet alone demonstrated significantly higher growth in marron compared with the growth of marron fed on other test diets. Supplementation of the basal diet with host origin *B. mycoides* significantly increased the intestinal bacterial population (3.399 ± 825 CFU g⁻¹ of gut) in marron compared with other diets. Organic selenium as Sel-Plex in Trypticase Soya Broth also confirmed that OS did not increase the amount of growth of *B. mycoides* and resulted in a lower intestinal bacterial population in the synbiotic diet-fed marron. In conclusion, synbiotic of OS and *B. mycoides* may improve a particular immune parameters of marron and to a lesser extent their growth.

Keywords: marron, synbiotic, *Bacillus mycoides*, organic selenium, immunity and growth

Introduction

Prebiotics and probiotics have been extensively used in aquaculture (Burr & Gatlin 2005; Denev, Staykov, Moutafchieva & Beev 2009; Ganguly, Paul & Mukhopadhayay 2010; Dimitroglou, Merrifield, Carnevali, Picchietti, Avella, Daniels, Güroy & Davies 2011; Kristiansen, Merrifield, Vecino, Myklebust & Ringø 2011; Merrifield & Zhou 2011). Prebiotics are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson & Roberfroid 1995; Ringø, Olsen, Gifstad, Dalmo, Amlund, Hemre & Bakke 2010). Prebiotics have shown beneficial effects on numerous aquatic animals (Zhou, Buentello & Gatlin 2010), and reviews of their potential use in aquaculture have been documented (Merrifield, Dimitroglou, Foey, Davies, Baker, Bogwald, Castex & Ringø 2010; Ringø et al. 2010; Merrifield & Zhou 2011). The prebiotics commonly used and evaluated in aquatic animals to date include inulin, fructooligosaccharides, mannanoligosaccharides (MOS), galactooligosaccharides, xylooligo-saccharides, arabinolxylooligosaccharides, isomaltooligosaccharides and GroBiotic-A (Ringø
et al. 2010), chitosan oligosaccharides (COS) and organic selenium (OS). In marron Cherax cainii (Austin 2002), the probiotics evaluated were MOS (Sang, Ky & Fotedar 2009; Sang & Fotedar 2010) and OS (Nugroho & Fotedar 2013b).

Probiotics, defined as live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002), have been recommended as alternatives for use as growth promoters, diseases control and for the safety of sustainable aquaculture (For reviews see: Ringo, Strom & Tabacheck 1995; Ringo & Gatesoupe 1998; Verschuere, Rombaut, Sorgeloos & Verstraete 2000; Irianto & Austin 2002; Austin 2006; Balcazar, Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Mu’azqib 2006; Farzanfar 2006; Das, Ward & Burke 2008; Sahu, Swarnakumar, Sivakumar, Thangaradjou & Kannan 2008; Denev et al. 2009; Ninawe & Selvin 2009; Qi, Zhang, Boon & Bossier 2009; Merrifield et al. 2010; Nayak 2010; Prado, Romalde & Barja 2010; Cerezuela, Meseguer & Esteban 2011; Kolndadacha, Adikwu, Okaeme, Atiribom & Mohammad 2011; Lara-Flores 2011; Martinez Cruz, Ibanez, Monroy Hermosillo & Ramirez Saad 2012; Ibrahim 2013; Lakshmi, Viswanath & Gopal 2013; Pandiyam, Balaraman, Thirunavukkarasu, George, Subaramaniyan, Manikkan & Sadayappan 2013) and more recently (Lazado, Marlowe & Cal pang 2014; Michael, Amos & Hussaini 2014; Newaj-Fyuzal, Al-Harbi & Austin 2014; Ghanbari, Caipang 2014; Balc & Norgren 2015; Hai 2015).

Probiotics and prebiotics generally have been studied separately (Li, Tan & Mai 2009; Cerezuela, Meseguer & Esteban 2011), European lobster Homarus gammarus (Tapia-Paniagua et al. 2001; Ambas et al. Aquaculture Research, 2016, 1–12), and more recently (Lazado, Marlowe & Cal pang 2014; Michael, Amos & Hussaini 2014; Newaj-Fyuzal, Al-Harbi & Austin 2014; Ghanbari, Caipang 2014; Balc & Norgren 2015; Hai 2015).

Marron, Cherax cainii (Austin, 2002) 10.83 ± 0.28 g were supplied by Marron Growers Association of Western Australia located in Northcliffe and Manjimup. The marron were acclimated to experimental tanks and feed for 2 weeks before the experiment commenced. During the acclimation, marron were fed a basal diet at 1.5% of total biomass per tank once a day at 17:00 hours.

Commercial feed was purchased from Glenn Forrest, (Glenn Forrest, Australia), with a composition of 26% crude protein, 9% crude fat and 5% crude ash. The commercial marron feed was homogenized using a blender to obtain a desirable pellet size for marron and for inclusion of B. mycoides, OS (Sel-Plex) and probiotic OS (Sel-Plex) before storing at 4°C. Bacillus mycoides was added at 10^6 CFU g^-1 of feed following the previous study (Ambas et al. 2013), whereas OS (Sel-Plex) was supplemented at 0.2 g kg^-1 of feed (Nugroho & Fotedar 2013b). The marron were fed the experimental diets for

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10 weeks at 1.5% of total biomass per tank, once per day in the late afternoon (Jussila 1997).

Experimental set up

The experimental tanks were 250-L cylindrical plastic tanks (80-cm diameter and 50-cm high) filled with freshwater and supplied with constant aeration. The tank was also equipped with a submersible thermostat set to 24°C. Water in each tank ran continuously at a rate of approximately 3 L min⁻¹ using recirculating biological filtration. Sufficient PVC pipes of appropriate diameters were placed into each tank as marron shelters. Following the acclimation, the marron were distributed equally into 12 experimental culture tanks at the density of 12 marron tank⁻¹, where each group was in triplicate.

Data collection

Total and differential haemocyte count

The THC and differential haemocyte count DHC (proportion of hyaline cell) of marron were measured on the final day of the feeding trial. Haemocyte sample preparation and calculation were done using an established method (Jussila, Jago, Tsvetnenko, Dunstan & Evans 1997; Fotedar 1998). Briefly, 0.5 mL of haemolymph withdrawn from the second last ventral segment of marron was placed into a haemocytometer (Neaubauer, Munich, Germany) immediately viewed under a camera equipped with a microscope, images were then taken for THC and DHC counts. For each treatment group, the procedure was repeated ten times using a different animal. The THC was calculated as

\[
\text{THC} = \left( \frac{\text{cells counted} \times \text{dilution factor}}{1000} \right) / \text{volume of grid (0.1 mm}^3). 
\]

The proportion of haemolymph cells (DHC) was also measured using established methods (Bancroft & Stevens 1977; Hai & Fotedar 2009). One drop of the mixture of anticoagulant and haemolymph was smeared onto a glass microscope slide and air-dried before fixing in 70% methanol for 10 min. The slides were stained in May–Grunwald and Giemsa for 10 min each. Identification of the haemocyte cell groups followed the criteria described by Bauchau (1981) and Johansson, Keyser, Sritunyalucksana and Soderhall (2000). On each slide, a total of 200 cells were counted and the percentage of a haemocyte group was calculated using the equation:

\[
\text{DHC} (\%) = \left( \frac{\text{Number of haemocytes cell type}}{\text{total haemocytes cells counted}} \right) \times 100 
\]

Haemolymph bacteria (Bacteraemia)

Assessment of bacteraemia was performed following the established procedure described by Fotedar, Tsvetnenko and Evans (2001) with a minor modification. The haemolymph was withdrawn into a sterile syringe and placed onto a sterile glass slide from which a 0.05-mL aliquot was smeared onto a blood agar (BA) plate. This technique was effective in removing bubbles of the haemolymph so that an accurate volume was smeared onto the plates. Subsequently, the plates were placed in a sterilized container before overnight incubation at 25°C. The total colony-forming units (CFU) for each plate and CFU mL⁻¹ were calculated on the basis of a total volume of 0.05 mL⁻¹ plate.

Haemolymph clotting time

Determination of haemolymph clotting time followed the established method (Fotedar et al. 2001; Jussila, McBride, Jago & Evans 2001). Briefly, the haemolymph of marron was withdrawn using a sterile syringe and dispensed into an Eppendorf tube. A 30-µL aliquot was quickly transferred and drawn into a capillary tube (Chase, Scientific Glass, Rockwood, TN, USA), then the tube was repeatedly inverted until the haemolymph stopped moving, which was noted as haemolymph clotting time (seconds).

Glutathione peroxidase

Sample preparation and determination of the GPx activity in marron tissue followed the established protocol (Rotruck, Pope, Ganther, Swanson, Hafeman & Hoekstra 1973). The marron tissue was diluted with physiological saline at a ratio of 1:1 and stored at 4°C until used. The GPx activity was expressed as micrograms of GSH consumed per minute per milligram of protein.

Intestinal bacterial density

Bacterial density in marron intestine was determined following the established protocol (Hai & Fotedar 2009). In brief, five marron of equal size from each treatment group were selected and rinsed in distilled water. Subsequently, the shells were cleaned with 70% alcohol then rinsed again
in distilled water to remove the external bacteria. Following dissection, the intestine of each marron was removed and weighed, then homogenized using a sterilized pestle and mortar. Subsequently, the homogenates were diluted serially with sterilized normal saline then lawn inoculated to nutrient agar (NA) plates prior to incubation for 24 h at 25°C. The total colony-forming units (CFU) for each plate and CFU mL\(^{-1}\) were calculated on the basis of a total volume of 0.05 mL plate\(^{-1}\) (Buller 2004).

**Effect of OS on Bacillus mycoides**

Sel-Plex (Alltech) does not dissolve in water, but does dissolve in NaOH. The recommended dose as a feed additive is 0.2 g kg\(^{-1}\). To determine whether OS (Sel-Plex) dispersed in water, or dissolved in 0.08% sodium hydroxide would affect the growth of *B. mycoides*, an experiment was performed by adding the Sel-Plex and solvent to media as treatments in triplicate as follows; (1) *B. mycoides* in TSB only, (2) *B. mycoides* in TSB with 0.2 g kg\(^{-1}\) OS (Sel-Plex), and (3) *B. mycoides* in sodium hydroxide added to TSB. To achieve the lowest concentration of sodium hydroxide which dissolved Sel-Plex 0.2 g kg\(^{-1}\) OS (Sel-Plex), a working solution of OS (Sel-Plex) in sodium hydroxide was prepared by a serial dilution to the desired concentration.

Prior to the experiment, pure *B. mycoides* was grown on BA plates and incubated overnight at 24°C, then emulsified in sterile normal saline to be used as an inoculum. Subsequently, 100 µL of the inoculum was added to each media of the three treatments then incubated 24 h at 25°C. Determination of *B. mycoides* density in each treatment was obtained by total bacterial count on BA plates (Buller 2004).

**Specific growth rate**

The specific growth rate (SGR) of marron was determined by measuring the average weight of 15 marron from each treatment group at the beginning and end of the feeding trial. The specific growth rate was calculated as follows:

\[
\text{SGR} \, (\%) = 100 \times (\ln W_t - \ln W_0)t^{-1}
\]

where SGR is the specific growth rate in weight (\(\%\ \text{g day}^{-1}\)), \(W_t\) is the weight of marron at measurement (\(t\)) and at the commencement (\(W_0\)), where \(t\) is experimental period (day).

**Survival rate (%)**

At the end of the experimental period, the number of marron in each tank was counted and survival rate was calculated using the following formula:

\[
\text{SR} = \left(\frac{N_t}{N_0}\right) \times 100
\]

where SR is the survival rate (%); \(N_t\) is the number of marron at time \(t\) and \(N_0\) is the number of marron at the commencement (0) respectively.

**Water quality**

The water quality in each tank was kept at optimum conditions for marron by performing water exchange at a rate of 10–15% of the total water volume twice a week, after siphoning out the faeces and uneaten feed. Several water quality parameters were monitored weekly including total ammonia and nitrite, which were measured using Calorimeter PR 1890, USA; temperature and pH using a digital pH/mV/C meter, Cyberscan pH300; Eutech instruments, Singapore, Singapore; and dissolved oxygen using a digital DO meter SM600, Milwaukee, Romania.

**Data analysis**

The data were analysed using SPSS statistical package version 22.0 for Windows and Microsoft Excel. Significant differences among treatment means were determined using one way analysis of variance (ANOVA). All significant tests were performed at \(P < 0.05\) level. The results were presented as means ± SE (standard error).

**Results**

**Immune competence**

Immune competences selected in this study (THC, DHC, bacteraemia, clotting time and GPx activity) are presented in Table 1 and Fig. 1. All immune parameters observed were significantly higher (\(P < 0.05\)) in supplement-fed marron compared with basal diet-fed marron.

Symbiotic use of *B. mycoides* and OS synergistically improve immunity particularly the GPx
enzyme activity of marron and to some extent THC and DHC (hyaline cell). In general, the synbiotic feed improved marron immunity significantly higher compared with OS and basal diet-fed marron but comparable to the single use of _B. mycoides_.

**Intestinal bacteria density**

At the termination of the feeding trial, the intestinal bacterial density (million g⁻¹ of gut) was significantly different (_P_ < 0.05). Marron fed _B. mycoides_ showed the highest intestinal bacterial density (3.399 ± 0.825 CFU g⁻¹ of gut) compared with basal diet-fed marron (519 ± 176 CFU g⁻¹ of gut) (Fig. 2).

**Effect of OS on _Bacillus mycoides_**

The growth (number of colonies) of _B. mycoides_ was measured by total bacterial count after 24 h of incubation and indicated that dissolved OS (Sel-Plex) in TSB media did not improve the growth of _B. mycoides_. In fact, the number of _B. mycoides_ colonies was significantly lower (186.6 × 10⁶ CFU mL⁻¹) in TSB media containing OS compared with a count of 382.6 × 10⁶ CFU mL⁻¹ in TSB media without OS. In addition, 0.08% of sodium hydroxide used as an OS solvent did not inhibit _B. mycoides_ growth, as the total colony count in sodium hydroxide prepared with TSB media was similar (366.6 × 10⁶ CFU mL⁻¹) to the total colony of _B. mycoides_ in TSB only.

**Specific growth rate**

Growth of marron fed various supplemented diets at the end of the feeding trial is presented in Table 2. The highest specific growth rate was detected in marron fed _B. mycoides_-supplemented diet. Although the lowest growth rate was observed in marron fed the basal diet, the growth rate was not significantly different to OS or synbiotic-fed marron.

**Survival rate (%)**

The survival rate of marron fed various supplemented diets at the termination of the feeding trial is presented in Fig. 3. No mortality was observed in tanks of marron fed _B. mycoides_, OS (Sel-Plex) or a combination of both, but deaths occurred in basal diet-fed marron.

**Water quality**

The water quality in this study was maintained within the optimum range for marron growth, as each tank was equipped with a bio-filter

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### Table 1 Immune competence (mean ± SE) of marron fed _Bacillus mycoides_, organic selenium (OS) and synbiotic diets

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th><em>B. mycoides</em></th>
<th>OS</th>
<th><em>B. mycoides</em> + OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC (10⁵ mL⁻¹)</td>
<td>2.858 ± 1.1c</td>
<td>9.471 ± 0.8a</td>
<td>4.162 ± 1.0b</td>
<td>8.129 ± 1.4a</td>
</tr>
<tr>
<td>Hyaline cell (%)</td>
<td>24.4 ± 3.2c</td>
<td>40.8 ± 4.2a</td>
<td>32.5 ± 3.1b</td>
<td>38.3 ± 2.9a</td>
</tr>
<tr>
<td>Bacteraemia (CFU mL⁻¹)</td>
<td>4020 ± 1.666c</td>
<td>668 ± 433a</td>
<td>2584 ± 832b</td>
<td>2576 ± 470b</td>
</tr>
<tr>
<td>Clotting time (s)</td>
<td>71.7 ± 6.9d</td>
<td>36.4 ± 6.8a</td>
<td>56.9 ± 5.6b</td>
<td>53.7 ± 12.6b</td>
</tr>
</tbody>
</table>

_B. mycoides_ was added at 10⁸ CFU g⁻¹ of feed, whereas OS was supplemented at 0.2 ppm kg⁻¹ of feed. Data in the same row having different superscripts indicates significantly different at 0.05 (n = 10; _P_ < 0.05).
recirculation system and regular water exchange was conducted. No significant difference was observed for the water quality in tanks among the treatments (Table 3).

**Discussion**

Probiotics and prebiotics have been proven to improve immunity and physiological aspects in various aquaculture species; hence studies on feed supplements are now focused on synbiotics to explore further synergetic effects in aquatic animals.

Total haemocyte count, proportion of hyaline cell (DHC), bacteraemia and haemolymph clotting time (Jussila et al. 1997; Sang et al. 2009), glutathione-S-transferase (GST) and GPx (Nugroho & Fotedar 2013a) have been successfully used as tools to evaluate immunity and health status in marron studies. The present study demonstrated that supplementation with either *B. mycoides*, OS (Sel-Plex), or their combination as a synbiotic significantly improved marron immune parameters, particularly GPx enzyme activity and THC. The significant increase in the GPx and THC in the synbiotic-fed marron compared with use of OS (Sel-Plex) alone or the basal diet-fed marron indicated a synergetic effect of the synbiotic on marron immunity. Previous studies using OS (Sel-Plex) also demonstrated improved marron THC.

Table 2 Specific growth rate (% day$^{-1}$) of marron fed *Bacillus mycoides*, organic selenium (OS) and synbiotic diets

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Basal diet</th>
<th>B. mycoides</th>
<th>OS</th>
<th>B. mycoides + OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight</td>
<td>10.83 ± 0.28</td>
<td>10.83 ± 0.28</td>
<td>10.83 ± 0.28</td>
<td>10.83 ± 0.28</td>
</tr>
<tr>
<td>Final weight</td>
<td>13.06 ± 1.52</td>
<td>14.29 ± 1.55</td>
<td>13.21 ± 1.57</td>
<td>13.71 ± 1.63</td>
</tr>
<tr>
<td>SGR</td>
<td>0.16 ± 0.05a</td>
<td>0.21 ± 0.07b</td>
<td>0.17 ± 0.06a</td>
<td>0.18 ± 0.04b</td>
</tr>
</tbody>
</table>

*B. mycoides* was added at $10^8$ CFU g$^{-1}$ of feed, whereas OS was supplemented at 0.2 ppm kg$^{-1}$ of feed. Data in the same row having different superscripts indicate significantly different at 0.05 ($n = 15$; $P < 0.05$).

**Figure 2** Intestinal bacterial population ($10^6$ CFU g$^{-1}$ of gut) of marron fed various diets ($n = 5$).

**Figure 3** Survival rate (%) of marron fed different diets at the termination of the feeding trial.
(Nugroho & Fotedar 2013a, 2015), and the use of *B. mycoides* (Ambas et al. 2013). Increased THC and improved immunity of aquatic animals fed a synbiotic diet has also been observed in shrimps *P. japonicus* (Zhang et al. 2011) and *L. vannamei* (Li et al. 2009), rainbow trout *O. mykiss* (Rodriguez-Estrada et al. 2009; Mehrabi, Firouzbakhsh & Jalafpour 2012), Japanese flounder *P. olivaceus* (Ye et al. 2011), sea cucumber *Apostichopus japonicas* (Zhang et al. 2010) and koi *Cyprinus carpio* (Lin, Mao, Guan, Luo, Luo & Pan 2012). However, synbiotic use of *B. subtilis* or *Pediococcus acidilactici* did not improve THC of shrimp, *L. vannamei* (Wongsasak, Chaijamrus, Kamkhong & Boonanuntanasarn 2015).

The haemocyte is responsible for destroying invasive microorganisms (bacteraemia) and foreign particles, thus greater THC contributes to better cellular immunity (Sang, Fotedar & Filer 2011). In Australia, bacteraemia has been associated with mortalities in crayfish (Eaves & Ketterer 1994; Edgerton & Owens 1999), as a result of exposure to environmental stressors (Jussila et al. 1997; Evans & Edgerton 2002; Sang et al. 2009). Once pathogens or foreign particles enter the haemocoel, the haemocyte initiates phagocytosis (Li, Yeh & Chen 2010), whereas in crayfish the phagocytosis is chiefly executed by hyaline cells (Johansson et al. 2000). In the present study, bacteraemia of probiotic-*, OS- and synbiotic-fed marron was significantly lower compared with basal diet-fed marron indicating that probiotic, OS and their synbiotic effectively reduces foreign particles in the haemolymph as a result of higher THC and proportion of hyaline cells.

Chronic bacteraemia results in immunosuppression, reduced growth rates in *Cherax quadricarinatus* (Edgerton & Owens 1999) and mortality, thus clearance of the bacterial pathogens in the circulating haemolymph is essential to maintain animal health. Sang et al. (2009) and Nugroho and Fotedar (2015) demonstrated that greater THC and a higher proportion of hyaline cells reduce bacteraemia in marron. Our previous study also confirmed a significant bacteraemia reduction in marron fed *B. mycoides* at 48 and 96 h post injection challenge test with the pathogen *V. mimicus* (Ambas et al. 2013). Chisholm and Smith (1995) evaluated antibacterial activity of four species of crustaceans and found that their haemocytes contained factors able to reduce the viable count of injected bacteria within 4 h.

The antioxidant enzymes, including GPx, which is a very potent antioxidant, protect the body from damage from oxidation by free radicals (Chiu, Hsieh, Yeh, Jian, Cheng & Liu 2010), which can cause cellular damage and oxidative stress (Parrilla-Taylor & Zenteno-Savin 2011). In this study, GPx was significantly higher in the muscle of synbiotic-fed marron (Fig. 1) compared with other test diets. Supplementation with OS (Sel-Plex) also significantly improved the antioxidant enzymes, GST and GPx, in marron haemolymph (Nugroho & Fotedar 2013a). In contrast, synbiotic use of *β-glucan* and *B. subtilis* in feed resulted in significantly lower superoxide dismutase (SOD) activity in shrimp *L. vannamei*, compared with *β-glucan* alone (Wongsasak et al. 2015).

The SGR of marron improved significantly when fed *B. mycoides*, OS (Sel-Plex) and their synbiotics in supplemented feeds compared with basal diet-fed marron. However, marron fed synbiotic feed was not significantly different to singular supplementation of either *B. mycoides* or OS (Sel-Plex). Improved growth using synbiotic feed has been demonstrated in rainbow trout *O. mykiss* fingerlings (Rodriguez-Estrada et al. 2009; Mehrabi et al. 2012), European lobster *H. gammarus* (Daniels et al. 2010), Japanese flounder *Paralichthys* (Ye et al. 2011) and Nile tilapia *Oreochromis niloticus* (Hassaan, Soltan & Ghonemy 2014).

The main purpose of using both prebiotics and probiotics in aquatic animals is to improve health, immunity and growth by stimulating commensal intestinal bacteria of the host. The feed supplementation of *B. mycoides* or OS (Sel-Plex) alone

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**Table 3** Nitrite (ppm), pH, temperature (°C) and dissolved oxygen (ppm) in tanks during trial

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th><em>B. mycoides</em></th>
<th>OS</th>
<th><em>B. mycoides</em> + OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite</td>
<td>0.045 ± 0.01*a</td>
<td>0.034 ± 0.01*a</td>
<td>0.035 ± 0.01*a</td>
<td>0.033 ± 0.01*a</td>
</tr>
<tr>
<td>pH</td>
<td>7.60 ± 0.05*a</td>
<td>7.62 ± 0.07*a</td>
<td>7.58 ± 0.05*a</td>
<td>7.65 ± 0.03*a</td>
</tr>
<tr>
<td>Temperature</td>
<td>23.27 ± 0.81*a</td>
<td>23.47 ± 0.62*a</td>
<td>23.65 ± 0.84*a</td>
<td>23.53 ± 0.43*a</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>6.21 ± 0.20*a</td>
<td>6.25 ± 0.18*a</td>
<td>6.24 ± 0.42*a</td>
<td>6.21 ± 0.23*a</td>
</tr>
</tbody>
</table>

Data in the same row having different superscripts indicate significantly different at 0.05 (\( n = 3; P < 0.05 \)). OS, organic selenium.
significantly improved intestinal bacterial density compared with the basal diet-fed marron; however, the probiotic use did not further improve the total bacterial population compared with single supplementation. The highest intestinal bacterial load of B. mycoides-fed marron compared with basal diet and other probiotic candidates was also demonstrated in the previous study using various probiotic sources (Ambas et al. 2013). Zhang et al. (2011) observed a significant increase in total bacterial counts of shrimp P. japonicus fed a probiotic diet. Microbiota of GIT can be considered as a metabolically active organ (Gaggia, Mattearelli & Biavati 2010), which provides an initial barrier to pathogen entry (Sugita, Tsunohara, Ohkashi & Deguchi 1988; Verschuere et al. 2000; Ramirez & Dixon 2003; Ringo, Olsen, Mayhew & Myklebustd 2003), thus its stability (density and diversity) is essential for the health of an organism (Rollo, Sulpió, Nardy, Silvi, Oropianesi, Caggiano, Cresci & Carnevali 2006; Denev et al. 2009).

The effect of the Sel-Plex, yeast-derived OS, on B. mycoides evaluated in this study demonstrated that density of B. mycoides was higher in OS-free TSB media compared with B. mycoides in TSB + OS media indicating that OS (Sel-Plex) did not improve the growth of B. mycoides. Cerezuela et al. (2011) reviewed symbiotic studies and concluded that dietary symbiotic effect is most likely dependent on the fish species, feeding dose and duration, and the type of prebiotics and probiotics symbiotic combinations. In shrimp, L. vannamei, a combination of β-glucan and P. acidilactici was better than β-glucan and B. subtilis in terms of SOD activity of the animal (Wongsasak et al. 2015).

Synergetic effect of B. mycoides and OS was observed on marron immunity (GPx and THC), but not on the growth rate. A similar result was observed in koi, C. carpio koi, using dietary COS and B. coagulans, which synergistically improved innate immunity and resistance, but did not improve the growth (Lin et al. 2012). Presumably this was due to the fact that the probiotic B. mycoides did not grow well on OS-added substrate and resulted in a lower intestinal bacterial population (Fig. 2), as each strain has substrate preference (Rustall & Maitin 2002). No symbiotic studies have examined in vitro the effect of pure prebiotics solely on the selected probiotics to determine whether the probiotics improve or inhibit the growth of the probiotics. This test is essential to avoid any adverse effects of the sybiotic feeds on the hosts as was found in sea bream, S. aurata L., where the combined administration of inulin and B. subtilis increased susceptibility to infection (Cerezuela et al. 2012).

Members of the Bacillus genus represent the majority of bacteria used as probiotics due to their physical and biological characteristics (Wang, Li & Lin 2008). The most widely used in symbiotic studies include B. coagulans (Lin et al. 2012), B. subtilis (Zhang et al. 2010, 2011; Geng et al. 2011; Cerezuela et al. 2012, 2013; Zhang, Yu, Tong, Tong, Dong, Xu & Wang 2014; Wongsasak et al. 2015), B. licheniformis (Zhang et al. 2011; Hassaan et al. 2014), B. megeterium (Li et al. 2009) and B. clausii (Ye et al. 2011). In the present study, B. mycoides was selected as it was of host origin and possessed a number of probiotic properties (Ambas, Buller et al. 2015) such as improved marron immunity against V. mimicus (Ambas et al. 2013) and improved GIT health status (Ambas, Fotedar et al. 2015). Overall, the present study suggested that B. mycoides and OS (Sel-Plex) contribute a synergetic effect on marron immunity particularly on oxidative enzyme activity (GPx) and the THC, but to a lesser extent on growth rate. In addition, an in vitro test revealed that dissolved OS (Sel-Plex) in TSB media did not improve B. mycoides growth.

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