Inhibitory activity of native probiotic *Bacillus vallismortis* IS03 against pathogenic *Vibrio harveyi* under *in vitro* and *in vivo* conditions in *Litopenaeus vannamei*

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Abstract

An excellent alternative for chemical antimicrobial agents to prevent disease in the shrimp aquaculture is the application of effective probiotics. The present study was evaluated the effect of *Bacillus vallismortis* IS03 as a native probiotic, isolated from digestive tract of *Litopenaeus vannamei* on pathogenic *Vibrio harveyi* under *in vitro* and *in vivo* circumstances. Co-cultivation of *V. harveyi* and *B. vallismortis* showed significantly (P<0.05) decreased the growth of *V. harveyi* in the treatment groups compared to the control. Cell-free extracts of *B. vallismortis* IS03 exhibited more appropriate antibacterial effects on replication of *V. harveyi*. The highest and lowest inhibitory effects were respectively shown in10^8 and 10^6 CFU ml^{-1} of *B. vallismortis* IS03 cell-free extracts. The probiotic potential of *B. vallismortis* IS03 was assessed through the groups of control and the experiments 10^6, 10^7 and 10^8 CFU ml^{-1} salt water once every 3 days from zoea1 process to end point of the study. The probiotic potential of *B. vallismortis* IS03 was assessed through the groups of control and the experiments 10^6, 10^7 and 10^8 CFU ml^{-1} salt water once every 3 days from zoea1 process to end point of the study. Shrimp survival was determined after 10 days of challenge with *V. harveyi* at 10^5 CFU ml^{-1} (for the first 5 days) and 10^7 CFU ml^{-1} (for the second 5 days).The cumulative mortality in the treatment with 10^8 CFU ml^{-1} of *B. vallismortis* IS03 reached 11.88% compared to 40.63% in the control group. At the end of the trial, total bacterial counts on TSA, total *vibrio* on TCBS were significantly (P<0.05) lower in the 10^8 CFU ml^{-1} treatment group. *Bacillus* counts on MYP agar in the treatment groups were significantly (P<0.05) higher than the control, also total bacterial counts was lower in the treatment groups, while, no *vibrio* were grown in the muscle tissues of shrimp treated with probiotics. It is concluded that 10^8 CFU ml^{-1} of probiotic, *B. vallismortis* IS03 has antibacterial efficiency against pathogenic *V. harveyi* at *in vitro* and *in vivo* conditions.

Keywords: Probiotic, *Bacillus vallismortis*, *Litopenaeus vannamei*, *Vibrio harveyi*

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Introduction

During the recent decades, the shrimp aquaculture industry has considerably developed as a main sector of food production. However, due to greater level of stocking of shrimp in hatcheries or rearing ponds resulting in entering the greater volume of protein and nutrients into the ponds, the environment of there is provided for growing the bacteria and other pathogens and their related infections transmission (Moriarty 1999).

Some *Vibrio* species are the main pathogens of reared shrimp. These bacteria are accountable outbreaks specifically early mortality syndrome (EMS) occurring in the last decade throughout the world, which showed mortalities up to 100% (Karunasagar & Malaty 1994). Microbial analysis of pond bottom and water samples of reared shrimp in Bushehr Province located in south of Iran indicating a higher presence of *Vibrio* spp. (%37/88) in comparison with *Bacillus* spp. (%27/27) in all of the ponds (Mirbakhsh, Akhavansepahy, Afsharnasab, Khanafari & Razavi 2013). The Gram positive bacteria, which typically found at low concentration approximate 20% of the total microbiota of pond bottom is not adequate for a well-protection environment against Gram negative pathogen infections (Swan & Singh 2009). In order to prohibit the aquatics to be prone to infections, use of antibiotics had been widespread in aquaculture throughout the world resulted in generations of antibiotic-resistance bacteria, which could be harmful for consumers. (Moriarty 1999). Probiotics are definite microbes that directly or indirectly are responsible for health advantages to the consumers when intake occurred in the appropriate amounts (Balcazar & Rojas-Luna 2007). The advantages of such additives containing enhancement of immunity level, improvement of food intake and digestion, unsaturated fatty acid production, anti-mutagenic and anticarcinogenic properties and ultimately growth-stimulating elements (Verschure, Sorgeloos & Verstreate 2000; Wang 2007). Therefore, the studies on application of environment-friendly probiotics in aquaculturve been extended (Gatesoupe 1999). Many investigations were made in case of shrimp culture to prevent of vibriosis in last decades (Rengpipat, Phianphak, Piyatiratitivorakul & Menasveta 1998a; Verschure et al. 2000; Dalmin, Kathiresan & Purushothaman 2001; Chytjanya, Karunasagar & Karunasagar 2002; Gomez- Gil, Roque & Velasco-Blanco 2002; Lee 2003; Meunpol, Lopinyosiri & Menasveta 2003; Alavandi, Vijayan, Santiago, Poornima, Jithendran, Ali & Rajan 2004; Gullian, Thompson & Rodriguez 2004; Balcazar & Rojas-Luna, 2007; Tseng, Ho, Huang, Cheng, Shiu, Chio & Lio 2009 and Mirbakhsh et al. 2013). *Bacillus* spp. are aerobic or voluntary anaerobic a saprophytic rod Gram-positive and non-pathogenic, spore-producing bacteria generally originate from each type of environment (Moriarty 1999; Gatesoupe 1999; Green, Wakeley, Page, Barnes, Baccigalupi, Ricca & Cutting 1999; Baruzzi, Quintieri, Morea & Caputo 2011). A few of these genus have probiotic potential to

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aggregate in gut and can activate against pathogenic microorganisms (Rengpipat et al. 1998; Vaseeharan & Ramasamy 2003; Immanuel, Citarasu, Sivaram & Palavesam 2007). For instances, the effect of probiotic, *Bacillus subtilis* E20, by adding to the rearing water on larvae shrimp (*L. vannamei*) at a certain concentration (10^9 CFU l^{-1}) which was improving the survival rate, development and overall immune status, was studied by Liu, Chiu, Shiu, Cheng & Liu (2010). Ziaei-Nejad, Habibi Rezaei, Azari Takami, Lovett, Mirvaghefi and Shakouri (2006) and Zokaeifar Saad, Doud, Harmin & Shakibzadeh (2009) documented the appropriate efficacy of some *Bacillus* spp. on improvement of digestive enzyme activity, better survivability and growth of *Fenneropenaeus indicus* at various biological phases.

The present study was aimed to investigate the inhibitory activity of *Bacillus vallismortis* IS03 as a native probiotic against pathogenic *V. harveyi* PTTC 1755 under *in vitro* and *in vivo* conditions.

Materials and Methods

Bacterial strains

Bacillus vallismortis strain IS03 (GenBank accession number JQ085958.1), previously isolated by the Iranian Shrimp Reaserch Center from digestive tract of *Litopenaeus vannamei* (Mirbakhsh et al. 2013) was used as probiotic strain. The virulent strain *of Vibrio, V. harveyi* PTTC1755 (GenBank accession number GU974342.1), was used as target bacteria (Mirbakhsh et al. 2014). All strains were stocked on tryptic soy agar (TSA, Merck, Germany) and cultured in tryptic soy broth (TSB, Merck, Germany) with 2.5% NaCl (w/v) and maintained in the laboratory under standard conditions. Samples were cultured into 2-L flasks at 30°C for 24h and the content of each flask was then centrifuged at 3000 × g for 10 min at 4°C, and washed in sterile normal saline solution (NSS) three times instantly before application. The purity of cultures was routinely checked during the investigation (Tseng et al. 2009).

Co-culture experiments

Bacillus vallismortis IS03 and *V. harveyi* were grown separately in TSB 2.5% in a shaking incubator at 30°C, overnight. Serial dilution *of B. Bacillus vallismortis* IS03 (10^6, 10^7 and 10^8 CFU ml^{-1}) was then prepared and initial cell density of approximately 10^5 CFU ml^{-1} of *Vibrio harveyi* was inoculated into *B. vallismortis* prepared solutions. All mixture were performed in triplicate. The co-culture flasks were incubated at 30°C, for 6-8 h. The samples exited from incubator every day to define *V. harveyi* concentration. The CFU ml^{-1} of *V. harveyi* were evaluated through the making 10-fold serial dilutions so that 0.1 ml from each dilution was incorporated into thiosulfate citrate bile salts sucrose agar plates (TCBS, Merck, Germany) (Vaseeharan & Ramasamy 2003).

The effect of cell-free extracts of *B. vallismortis* IS03 on *V. harvey*i

Bacillus vallismortis IS03 was cultured in TSB and then used as starter for inoculation to 50 ml of TSB in the same three mixture at a preliminary cell concentration of 10^6-10^8 CFU ml^{-1}. The flasks were incubated at 30°C in

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shaking incubator (150 rev min^{-1}) (JSSI-200 CL JSR Inc., Korea), the samples were daily checked in order to determine the number of bacteria by spread plate count method. The aseptic screened supernatant solution (2 ml) was assessed by adding 1 ml of supernatant to 1 ml of fresh TSB in test tubes and incorporated it 0.1ml of *V. harveyi*, producing approx.10^5 CFU ml^{-1}. Controls were made by inoculating The 0.1ml *V. harveyi* was added into 2 ml of TSB without *B. vallismortis* IS03 cell-free extracts. Each mixture was assessed in triplicate and the growth of the *V. harveyi* screened at 600 nm using a spectrophotometer (UV-Vis 6800 Jenway Inc., England) (Vaseeharan & Ramasamy 2003).

Rearing of shrimp

Litopenaeus vannamei nauplii were obtained from unilateral eyestalk ablated-females (average weight 40 g) in a commercial shrimp hatchery in the Bushehr Province of Iran. In this study, we were stocked at density of 100 larvae L^{-1} in three sets of 300-L fiberglass tanks in triplicates with 250 L seawater which was sand-filtered and treated with UV radiation. After filtration, the salinity was reduced to 30 ppt using fresh water. The water temperature was constant at 30 ± 1°C during the study. The pH of the sea water was 7.8-8.3. Water replacement was accomplished when the larvae development to the postlarvae stage was observed. Usually, 30% of the tank water was exchanged, daily. Four concentrations of probiotic were assigned as control, 10^6, 10^7 and 10^8 CFU ml^{-1} salt water and applied 2 times per week from zoea1 process to end point of the study. During the study, shrimps were fed four times a day. For the first feeding time, shrimp were fed diatoms (*Chaetoceros* spp.) at a concentration of 4000 Cells ml^{-1} from the zoea1 phase to the postlarvae. For the last 3 feeding times, shrimp were fed dried shrimp flakes which screened through a mesh of 250 µm, 200 µm, and 150 µm, at the zoea to postlarval developmental phases, respectively. The share of dried shrimp flaks for each tank differed from 0.2 to 1 gram (Liu et al. 2010).

Challenge test

For the pathogen challenge test, 1200 healthy *L. vannamei*, PL20 (100 postlarvae for each tank), were selected and challenged with the pathogenic *V. harveyi* for 10 days which following the method of Holt et al. (1994). Postlarvae of each group were challenged with a suspension of *V. harveyi* at the concentration of approx. 10^5 CFU ml^{-1} for the first 5 days (Austin et al. 1995). After 5 days, the postlarvae were re-challenged with the approx. 10^7 CFU ml^{-1} of *V. harveyi* until end of the study (Rengpipat et al. 1998a). During this evaluation, dead shrimps were exclude every 24 h, and the survivability of the postlarvae was documented every other day for each tank. At the end of the study, the accumulated mortality of the shrimp was recorded. After the challenge test, total heterotrophic bacteria, *Bacillus* and *vibrio* count from the tissue samples and water were enumerated by inoculated the samples on TSA, Mannitol-egg yolk-polymyxin agar (MYP agar) and TCBS agar (Merck, Germany), respectively based on the methods recommended by Immanuel et al. (2007). The study was carried

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out with the groups in triplicates and incubated at 30°C for 24 h. The bacterial colonies were then counted and recorded.

Statistical analysis

The data were analyzed using the SPSS software version no. 26 (SPSS Inc., Chicago, IL, USA). The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferoni multiple tests. The tests were significant at 0.05.

Results

Co-culture experiments

The results showed that the growth of pathogenic *V. harveyi* was inhibited by different levels of *B. vallismortis* IS03 (10^6- 10^8 CFU ml^{-1}) (Fig.1). The treatment groups were significantly ($P<0.05$) decreased the growth of *V.harveyi* during four days, other than the first day at concentrations of 10^6 and 10^7 CFU ml^{-1}). By increasing concentrations of *B. vallismortis* IS03, especially in the first 48 hours, the inhibitory activity was increased. Co-culture experiment results indicated that when the concentration of *B. vallismortis* IS03 wnt up, the growth of *V.harveyi* was controlled under *in vitro* situation.

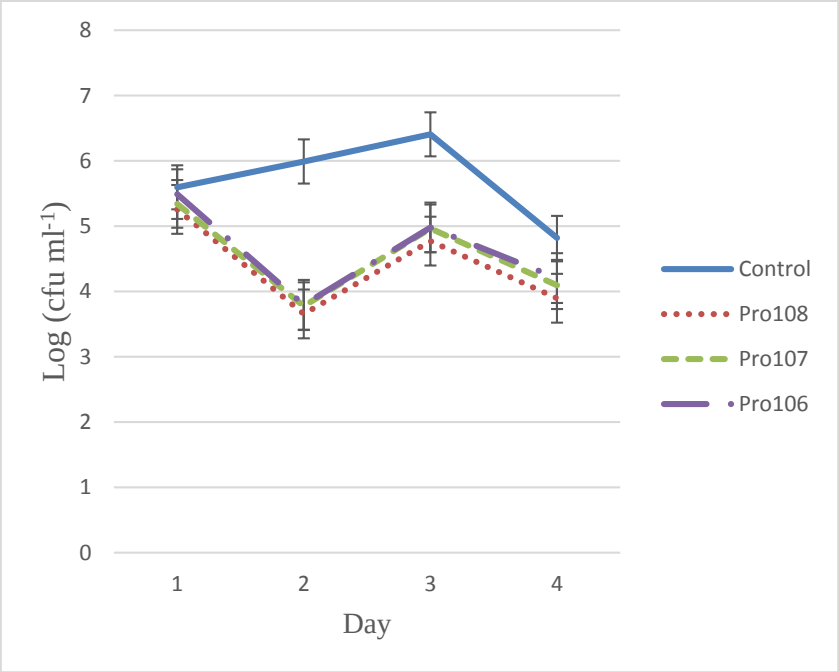

Figure 1. Growth pattern of *V. harveyi* with and without *B. vallismortis* IS03 at different initial concentrations (colony-forming units; CFU).

Effect of *B. vallismortis* IS03 cell-free extracts

Our results indicated that cell-free extracts of *B. vallismortis* IS03 can prevent the growth of *V. harveyi* in the liquid medium. The highest and lowest inhibitory effect was respectively at the concentration of 10^8 CFU ml^{-1} and 10^6 CFU ml^{-1}. *B. vallismortis* IS03 cell-free extracts. On the 2nd day, the growth of *V.harveyi* was remarkably inhibited when it was compared to the control group (Fig.2).

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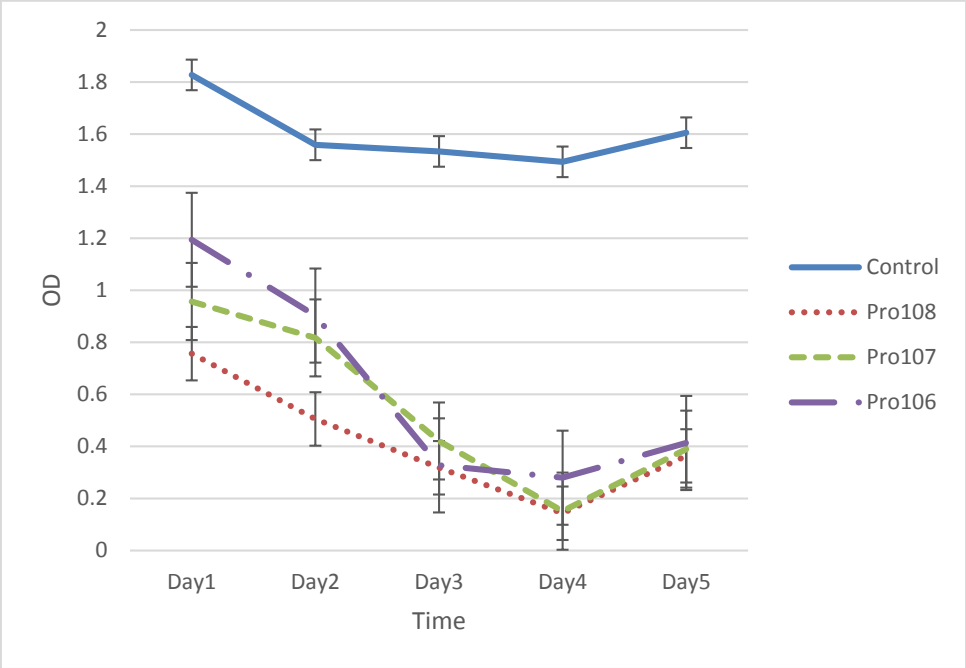

Figure 2. Growth of *V. harveyi* with and without cell-free extracts of *B. vallismortis* IS03 extracted by different cell densities.

Probiotic treatment and *V.harveyi* challenging study of shrimp

Experimental infection of shrimp and probiotic treatment showed that the *B. vallismortis* IS03 probiotic reduced mortality and thus increased shrimp survival against infection with pathogenic *V. harveyi* under *in vivo* conditions. The cumulative mortality of infected *L. vannamei* was not treated with *B. vallismortis* IS03 (control) reached 40.63% on the 10th day after infection by *V. harveyi*, while in the treatments of 10^6, 10^7 and 10^8 CFU ml^{-1} were 31.25%, 18.75% and 11.88%, respectively (Fig.3). No mortality was found in control group which were not challenged to *V. harveyi*. The first killed shrimp in the control, 10^6, 10^7 and 10^8 CFU ml^{-1} treatments were doccumented at 1st, 2nd, 3rd and 4th day respectively. After 5 days, by adding *V. harveyi* (10^7 CFU ml^{-1}) in tanks water, the mortality rate was increased in all groups. The mortality of postlarvae in the control and 10^6 CFU ml^{-1} treatments were higher than the 10^7 and 10^8 CFU ml^{-1} concentrations. Ultimately, the mortality rate of postlarvae for the control group showed significantly ($P<0.05$) greater than those of treatments.

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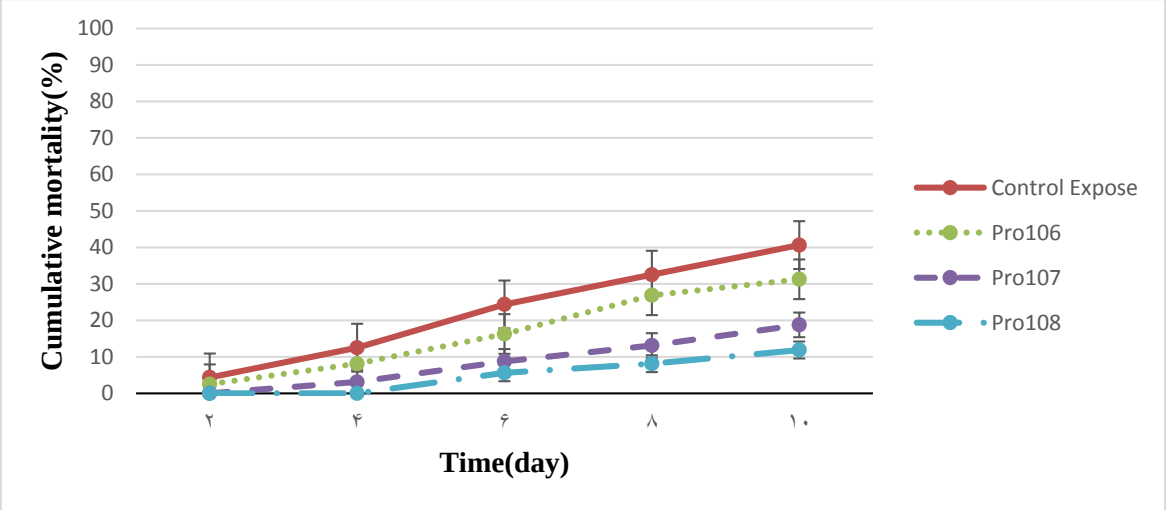

Figure 3. Cumulative mortality (%) of *Litopenaeus vannamei* postlarvae (PL30) during challenge test against shrimp pathogen *V. harveyi*.

At the end of experimental period, total bacteria, *Bacillus*, and *vibrio* populations were enumerated. Total bacterial counts on TSA, total *vibrio*, on TCBS were significantly ($P<0.05$) lower in the 10^8 CFU ml^{-1} treatment group than the control. *Bacillus* counts on MYP agar in the treatment groups were significantly ($P<0.05$) higher than the control (Table 1).

Table 1. Bacterial counts (log CFU ml^{-1}) in the *L.vannamei* postlarvae (PL30) rearing tanks water after challenging with *V. harveyi*

Bacterial counts (log CFU ml^{-1})	**control**	**10^6**	**10^7**	**10^8**
Total Bacteria count	5.35 ± 0.19^{a}	5.34 ± 0.17^{a}	5.30 ± 0.23^{a}	5.14 ± 0.09^{b}
Bacillus spp.	0.00 ± 0.00^{c}	3.45 ± 0.43^{b}	4.28 ± 0.36^{a}	4.51 ± 0.05^{a}
Total vibrio count	5.33 ± 0.24^{a}	4.89 ± 0.13^{b}	4.71 ± 0.07^{b}	4.66 ± 0.11^{b}

Different superscript letters are representing significant difference ($P<0.05$)

Moreover, total bacteria count, *Bacillus*, and *vibrio* counts in the muscle tissues of *L.vannamei* postlarvae (PL30), was determined after 10 days of the challenge test (Table 2). Total bacterial counts was lower in the treatment groups, while no *vibrio* bacteria were grown in the muscle tissues of shrimp treated with probiotics.

Table 2. Bacterial counts (log CFU ml^{-1}) in the muscle tissues of *L.vannamei* postlarvae (PL30) after challenging with *V.harveyi*

Bacterial counts (log CFU ml^{-1})	**control**	**10^6**	**10^7**	**10^8**
Total Bacteria count	6.01 ± 0.43^{a}	5.25 ± 0.51^{b}	5.23 ± 0.72^{b}	5.12 ± 0.36^{b}
Bacillus spp.	0.00 ± 0.00^{b}	3.57 ± 0.47^{a}	3.40 ± 0.29^{a}	3.24 ± 0.18^{a}
Total vibrio count	4.14 ± 0.06^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

Means in the same row with different superscripts are significantly different ($P<0.05$).

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Discussion

The beneficial effects of certain bacterial application in aquaculture have been previously addressed. The application of probiotics to promote the survival, growth performance, immunity level, and disease resistance of reared shrimp (Rengippat 2000 & Farzanfar 2006). The present study showed that by using *B. vallismortis* IS03, the growing of pathogenic *V. harveyi* under *in vivo* and *in vitro* conditions would be inhibited. Moreover, the co-culture experiments determined that the inhibitory action was a dose dependent process. Therefore, 10^8 CFU ml^{-1} concentrations of probiotics had the inhibitoriest effects on *V. harveyi*. This study showed that the antagonistic level must be existent at higher levels than those of the pathogens. It was shown that the replication of pathogenic *V. harveyi* in Monodon was significantly prohibited by the probiotic efficacy of *B.subtilis* BT23 both *in vitro* and *in vivo* situations. Moreover, they found an improvement in the disease shrimps and also a reduction of 90% accumulated mortality when juvenile of black tiger shrimp were challenged to *B.subtilis* BT23 isolated from shrimp culture ponds before challenging to *V. harveyi* (Vaseeharan & Ramasamy 2003). In a similar study, Rengpipat et al. (1998a) documented that incorporation with *Bacillus* S11, which had shown its prohibition outcome in vitro against *V. parahaemolyticus* and *V. harveyi*, resulted in better survival of *P. monodon* challenged with pathogenic luminescent bacteria. In this study, shrimp that exposed to pathogenic *V. harveyi* showed significant ($P<0.05$) reduction in cumulative mortalities in probiotic treated groups compared to the control group, however, no significant difference ($P>0.05$) were observed between 10^7 and 10^8 CFU ml^{-1} treatments. In addition, the results of culturing rearing water and the muscle samples plate culturing showed that the *B. vallismortis* IS03 can reduce total bacteria and total *Vibrio* counts. Immanuel et al. (2007) also reported a relative reduction in total *Vibrio* and total bacterial counts in the treatment groups compared to the control, in treating *P. monodon* postlarvaes and explained that the probionts will overwhelm the growth of the pathogenic bacteria by the reasonable elimination standard. The survival rate was 94.3% and 26.3% in postlarvae treated with probiotic and control, respectively.

Ajitha et al. (2004) reported an improved in the survival of shrimp *P. indicus* (56 to 72%) when *Lactobacillus* probiotic supplemented in the feeding of the groups challenged with *V. alginolyticus*. Rengpipat et al. (1998a) reported that, *Bacillus*, applied as a probiotic in *P. monodon* was able to aggregate both in pond water and the shrimp gut; the *Bacillus* also was able to replace with *Vibrio* spp. in the digestive tract of the shrimp, resulted in increase the survivability of shrimp.

Previous studies have suggested that the antibacterial efficiency of *Bacillus* could be due to either changes of pH *in vitro*, application of vital nutrients, or production of volatile combinations (Gullian et al. 2004; Chaurasia, Pandey, Palni, Trivedi, Kumar & Colvin 2005;

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Yilmaz, Soran & Beyatli 2006). In addition, several studies have documented that *Bacillus* excreted polypeptide with antibiotic properties, such as bacitracin, gramicidin S, polymyxin, and tyrotricidin (Tyrothricin), which are antagonist against a couple of Gram-positive and Gram-negative bacteria (Morikawa, Ito & Imanaka 1992; Perez, Suarez & Castro 1993; Drablos, Nicholson & Ronning 1999). Although the prohibition mechanism of the interface was not considered in this study, however, it seem logical that mechanism of *B. vallismortis* IS03 is performed according to reasonable elimination of the pathogen, because the bacterial evaluation showed that the occurrence of *B. vallismortis* in the shrimp tissues and pond water at the end of this trial. In conclusion, administration of the probiotic, *B. vallismortis* IS03 revealed a noticeable prophylactic effect against the growth of *V. harveyi* at both *in vitro* and *in vivo* conditions. It is therefore suggested that 10^8 CFU ml^{-1} concentration of this probiotic, add to rearing water of shrimps once every 3 days for better yields.

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Conflicts of interest

None of the authors has any conflicts of interest to declare.

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فعالیت مهاری پروبیوتیک بومی *Bacillus vallismortis* IS03 در برابر عامل بیماری زا *Vibrio harveyi* تحت شرایط درون‌تنی و برون‌تنی در میگوی *Litopenaeus vannamei*

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چکیده

یک جایگزین مناسب برای مواد شیمیایی با خاصیت ضد میکروبی به‌منظور جلوگیری از ابتلا به بیماری در میگو، استفاده از پروبیوتیک‌های مؤثر است. مطالعه حاضر اثر *Bacillus vallismortis* IS03 را به عنوان یک پروبیوتیک بومی جدا شده از دستگاه گوارش *Litopenaeus vannamei* در برابر عامل بیماری زای *Vibrio harveyi* در شرایط درون تنی و برون تنی مورد ارزیابی قرار داد. کشت توام ویبریو هاروئی و باسیلوس والیسمورتیس به طور معنی‌داری کاهش رشد ویبریو هاروئی در گروه های تیمار را نسبت به گروه شاهد نشان داد ($P<0.05$). عصاره بدون سلول از باسیلوس والیسمورتیس اثرات ضد باکتریایی مناسب‌تری در مقابل تکثیرویبریو هاروئی به نمایش گذاشت. بیشترین و کمترین اثر مهاری به ترتیب در عصاره های بدون سلول باسیلوس والیسمورتیس در غلظت‌های 10^8 و 10^6 کلنی در میلی‌لیتر مشخص گردید. پتانسیل پروبیوتیکی باسیلوس والیسمورتیس از طریق گروه‌های کنترل و تیمارهای 10^6 ، 10^7 و 10^8 کلنی در میلی لیتر آب نمک با انجام یک بار نمونه باری در هر ۳ روز از فرآیند zoea1 تا نقطه پایان صورت گرفت. زنده مانی میگو در چالش با ویبریو هاروئی در غلظت 10^5 کلنی در میلی لیتر پس از ۱۰ روز (برای ۵ روز اول) و در غلظت 10^7کلنی در میلی لیتر (برای ۵ روز دوم) تعیین شد. تلفات تجمعی در درمان با غلظت 10^8 کلنی در میلی لیتر از باسیلوس والیسمورتیس به ٪۱۱/۸۸ در مقابل گروه گروه شاهد (٪۴۰/۶۳) رسید . در پایان کارآزمایی، تعداد کل باکتریها و تعداد ویبریو کل شمارش شده به طور معنی داری ($P <0.05$) در گروه با غلظت 10^8 باکتری کمتر بود. شمارش باسیلوس ها در گروههای تیمار به طور معنی داری بیشتر از گروه شاهد بود ($P<0.05$) آن‌چنان که تعداد باکتری‌ها در گروه‌های تیمار کمتر گردید. این در حالی است که هیچ مقدار از ویبریو در بافت‌های عضلانی میگوهای تحت درمان با پروبیوتیک رشد ننمود. لذا اینچنین نتیجه گرفته می شود که غلظت 10^8 باکتری پروبیوتیک باسیلوس والیسمورتیس (ISO3) دارای بازده ضد باکتریایی در مقابل عامل بیماری‌زای هاروی در در هر دو شرایط برون تنی و درون تنی میگوی پا سفید وانمی است.

واژگان کلیدی: پروبیوتیک، *Bacillus vallismortis* ,*Litopenaeus vannamei* ,*Vibrio harveyi*

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