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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

excellent alternative for chemical An 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 in 108 and 106 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 10⁶, 10⁷ and 10⁸ CFU ml⁻¹ salt water once every 3 days from zoeal process to end point of the study.

*Correspondence M. Mirbakhsh, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran (e-mail: maryam.mirbakhsh@gmail.com). The probiotic potential of B. vallismortis IS03 was assessed through the groups of control and the experiments 10⁶, 10⁷ and 10⁸ 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 10⁵ CFU ml⁻¹ (for the first 5 days) and 10⁷ CFU ml⁻¹ (for the second 5 days). The cumulative mortality in the treatment with 108 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 10⁸ 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 10⁸ 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 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 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, antimutagenic 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 Phianphak, (Rengpipat, 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, sporeproducing 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

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 (10⁹ 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 Fenneropenaeus indicus 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 (10⁶, 10⁷ and 10⁸ CFU ml⁻¹) was then prepared and initial cell density of approximately 10⁵ 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 10⁶-10⁸ CFU ml⁻¹. The flasks were incubated at 30°C in

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.10⁵ 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 sandfiltered 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 accomplished when development to the postlarvae stage was observed. Usually, 30% of the tank water was exchanged, daily. Four concentrations of probiotic were assigned as control, 10⁶, 10⁷ and 10⁸ CFU ml⁻¹ salt water and applied 2 times per week from zoeal 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. 10⁵ CFU ml⁻¹ for the first 5 days (Austin et al. 1995). After 5 days, the postlarvae were rechallenged 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

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.

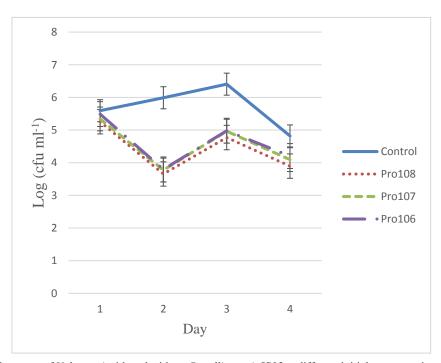


Figure 1. Growth pattern of *V. harveyi* with and without *B. vallismortis* IS03 at different initial concentrations (colony-forming units; CFU).

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 10⁸ CFU ml⁻¹ and 10⁶ 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).

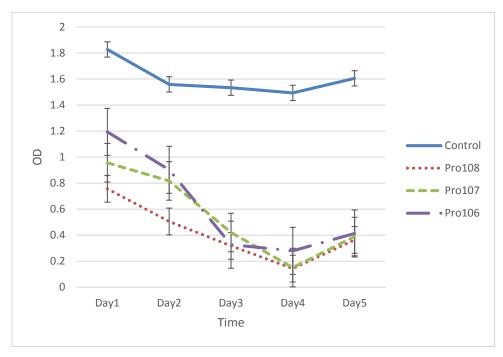


Figure 2. Growth of V. harveyi with and without cell-free extracts of B. vallismortis IS03 extracted by different cell densities.

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 10⁶, 10⁷ and 10⁸ 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.

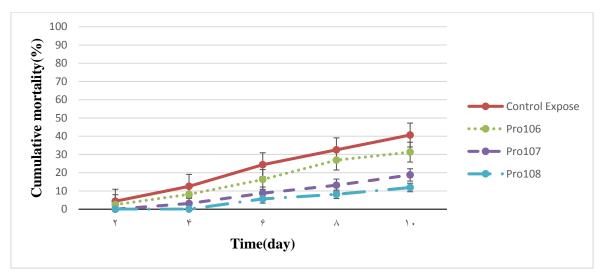


Figure 3. Cumulative mortality (%) of *Litopenaeus vannamei* postlarvae (PL30) during challenge test against shrimp pathogen *V. harveyi*.

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.vanname*i postlarvae (PL30) rearing tanks water after challenging with *V. harveyi*

Bacterial counts (log CFU ml ⁻¹)	control	10 ⁶	107	108
Total Bacteria count	5.35 ± 0.19^{a}	$5.34 \pm 0.17^{\mathrm{a}}$	5.30 ± 0.23^a	5.14 ± 0.09^{b}
Bacillus spp.	$0.00\pm0.00^{\mathrm{c}}$	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.vanname*i postlarvae (PL30) after challenging with *V.harveyi*

Bacterial counts (log CFU ml ⁻¹)	control	10^{6}	107	108
Total Bacteria count	$6.01\pm0.43^{\rm a}$	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).

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, 10⁸ 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. harvevi 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 compared to the control group, however, no significant difference (P>0.05) were observed between 10⁷ and 10⁸ 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;

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 Gramnegative 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 10⁸ 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.

References

Ajitha S., Sridhar M., Sridhar N., Singh I.S.B. & Varghese V. (2004) Probiotic effects of lactic acid bacteria against Vibrio alginolyticuys in Penaeus (Fenneropenaeus) indicus (H. Milne Edwards). Asian Fisheries Science 17, 71-80.

Alavandi S.V., Vijayan K.K., Santiago T.C., Poornima M., Jithendran K.P., Ali S.A. & Rajan J.J.S. (2004) Evaluation of Pseudomonas spp. PM 11 and Vibrio fluvialis PM 17 on immune indices of tiger shrimp, Penaeus monodon. Fish & Shellfish Immunology 17, 115-120.

Austin B., Stuckey L.F., Robertson P.A.W., Effendi I., Griffith D.R.W. (1995) A probiotic strain of Vibrio alginolyticus effective in reducing diseases caused by Aeromonas salmonicida, Vibrio anguillarum and Vibrio ordalii. Journal of Fish Diseases 18(1), 93-96.

Balcazar J.L. & Rojas-Luna T. (2007) Inhibitory activity of probiotic Bacillus subtilis UTM126 against Vibrio species confers protection against vibriosis in juvenile shrimp (Litopenaeus vannamei). Current microbiology 55, 409-412.

Baruzzi F., Quintieri L., Morea M. & Caputo L. (2011) Antimicrobial compounds produced by Bacillus spp. And applications in food. Science against microbial pathogens: Communicating Current Res Technological Advances A. Mendez- Vilas (Ed.), 1102-1111.

Chaurasia B., Pandey A., Palni L.M.S., Trivedi P., Kumar B. & Colvin N. (2005) Diffusible and volatile compounds produced

by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*. *Microbiological research* 160, 75-81.

Chio C-H., Guu Y-K., Pan T-M. & Cheng W., (2007) Immune responses and gene expression in white shrimp; *Litopenaeus vanamme*i, induced by *Lactobacillus plantarum*. *Fish Shellfish Immune* 31, 196-201.

Chun J., Lee J-H., Jung Y., Kim S., Kim B.K. & Lim Y.W. (2007) Ez Taxomn: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *International journal of systematic and evolutionary microbiology* 57, 2259-2261.

Chytjanya R., Karunasagar L. & Karunasagar I. (2002) Inhibition of shrimp pathogenic vibriosis by a marine Pseudomonas 1-2 strain. *Aquaculture* 208 (1-2), 1-10.

Dalmin G., Kathiresan K. & Purushothaman A., (2001) Effect of probiotic on bacterial population and health status of shrimp in culture pond ecosystem. *Indian J Exp Biol*, 39(9), 939 -942.

Drablos F., Nicholson D. & Ronning M. (1999) EXAFS study of zinc coordination in Bacitracin A. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* 1431, 433-442.

Farzanfar A. (2006) The use of probiotics in shrimp aquaculture. *FEMS Immunology & Medical Microbiology 48*(2), 149-158.

Gatesoupe F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* 180, 147-165.

Gomez-Gil B., Roque A. & Velasco-Blanco G. (2002) Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri*, *Aquaculture* 211(1-4), 43-48.

Green D.H., Wakeley P.R., Page A., Barnes A., Baccigalupi L., Ricca E. & Cutting S.M. (1999) Characterization of two Bacillusprobiotics. *Appl. Environ. Microbiol* 65(9), 4288-4291.

Gullian M., Thompson F. & Rodriguez J. (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture* 233, 1-14.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. & Williams S.T. (1994) Bergey's Manual of Determinative Bacteriology, 9th ed. Williams and Wilkins, Baltimore, pp. 260-274.

Immanuel G., Citarasu T., Sivaram V. & Palavesam, A. (2007) Delivery of HUFA probionts and biomedicine through bioencapsulated *Artemia* as a means to enhance the growth and survival and reduce the pathogenesity in shrimp *penaeus monodon* postlarvae. *Aquaculture International* 15, 137-152.

Karunasagar Y., Pai R. & Malaty G.R. (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture* 128, 203-209.

Kongnum K. & Hongpattarakere T. (2012) Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*. *Fish & Shellfish Immunology* 32, 170-177.

Lee C-S. (2003). Biotechnological advances in finish hatchery Production: a review. *Aquaculture* 227 (1-4), 439-458.

Liu K-F., Chiu C-H., Shiu Y-L., Cheng W. & Liu, C-H. (2010) Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vanammei* larvae. *Fish & Shellfish Immunology* 28, 837-844.

Meunpol O., Lopinyosiri K. & Menasveta P. (2003) The effects of ozone and probiotics on the survival of black tiger shrimp (*Penaeus monodon*). *Aquaculture* 220, 437-448.

Mirbakhsh M., Akhavan sepahy A., Afsharnasab M., Khanafari A. & Razavi M.R. (2014) Molecular identification of *Vibrioi harvey*i from larval stage of pacific white shrimp (*Litopenaeus vanammei*) Boone (Crustacea; Decapoda) by polymerase chain reaction and 16S rDNA Sequancing, *Iranian Journal of Fisheries Sciences* 13(2), 384-393.

Mirbakhsh M., Akhavansepahy A., Afsharnasab M., Khanafari A. & Razavi M.R. (2013) Screening and evaluation of indigenous bacteria from the Persian Gulf as a probiotic and biocontrol agent against Vibrio harveyi in

Litopenaeus vannamei post larvae. Iranian Journal of Fisheries Sciences 12(4), 873-886.

Moriarty D.J.W. (1999) Disease control in shrimp aquaculture with probiotic bacteria. Paper presented at the 8th Intertational Symposium on Microbial Ecology. Halifax, Canada.

Morikawa M., Ito M. & Imanaka T. (1992) Isolation of a new surf- actin producer *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulator gene, psf-1. *Journal of fermentation and bioengineering* 74, 255-261.

Perez C., Suarez C. & Castro GR. (1993) Antimicrobial activity determined in strains of *Bacillus circulans* cluster. *Folia microbiologica* 38, 25–28.

Rengpipat S., Phianphak W., Piyatiratitivorakul S. & Menasveta P. (1998a) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167, 301–313.

Rengpipat S., Rukpratanporn S., Piyatiratitvorakul S. & Menasaveta P. (2000) Immunity enhancement in black tiger shrimp (penaeus monodon) by a probiont bacterium (Bacillus S11). Aquaculture 191 (4), 271-288.

Swain S.M. & Singh C. (2009) Inhibitory activity of probiotic *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 against Vibriosis in shrimp. *World Journal of Microbiology and Biotechnology* 25, 697-703.

Tseng D-Y., Ho P-L., Huang S-Y., Cheng S-C., Shiu Y-L., Chio C-H. & Lio C-H. (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vanamme*i, by the probiotic, *Bacillus subtilis* E20. *Fish Shellfish Immunology* 26, 339-344.

Vaseeharan B. & Ramasamy P. (2003) Control of pathogenic *Vibrio* spp. by *Bacilus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *penaeus monodom. Letters in applied microbiology* 36(2), 83-87.

Verschure LRG, Sorgeloos P. & Verstreate W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and molecular Biology Reviews* 64, 655-671.

Wang Y-B. (2007) Effect of probiotics on growth performance and digestive enzyme

activity of the shrimp *Penaeus vanammei*. *Aquaculture* 269, 259-294.

Yilmaz M., Soran H. & Beyatli Y. (2006) Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiological research* 161, 127-131.

Ziaei-Nejad S., Habibi Rezaei M., Azari Takami Gh., Lovett L.D., Mirvaghefi A.R. & Shakouri M. (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* 252, 516-524.

Zokaeifar H., Saad C.R.B, Doud H.M., Harmin S. A. & Shakibzadeh S. (2009) Effect of *Bacillus subtilis* on the growth and survival rate of shrimp (*Litopenaeus vanname*). *African Journal of Biotechnology* 8 (14), 3369-3376.

فعالیت مهاری پروبیوتیک بومی Bacillus vallismortis IS03 در برابر عامل بیماری زا Vibrio harveyi تحت شرایط درون تنی و برون تنی در میگوی

Litopenaeus vannamei

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چكىدە

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 ISO3 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 ۱۰^۸ و ۱۰^۶ کلنی در میلیلیتر مشخص گردید. پتانسیل پروبیوتیکی باسیلوس والیسمورتیس از طریق گروههای کنترل و تیمارهای ۱۰٬ ۱۰٬ و ۱۰۸ کلنی در میلی لیتر آب نمک با انجام یک بار نمونه باری در هر ۳ روز از فرآیند zoeal تا نقطه پایان صورت گرفت. زنده مانی میگو در چالش با ویبریو هاروئی در غلظت ۱۰^۵ کلنی در میلی لیتر پس از ۱۰ روز (برای ۵ روز اول) و در غلظت ۱۰٬ کلنی در میلی لیتر (برای ۵ روز دوم) تعیین شد. تلفات تجمعی در درمان با غلظت ۱۰^۸ 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 ان چنان (P<0.05) باکتری کمتر بود. شمارش باسیلوس ها در گروههای تیمار به طور معنی داری بیشتر از گروه شاهد بود 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 ۱۰^۸ 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 یا سفید وانمی است.

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

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