Putative Bacterial Flora Assessment in Juvenile *Litopenaeus vannamei* as Probiotics by using Antibacterial Activity Tests

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The objective of this study was to find an effective probiotic of putative bacterial flora, using *in vitro* antagonism test in *Litopenaeus vannamei*. Twelve groups of putative bacterial flora, isolated from juvenile *Litopenaeus vannamei*Persian Gulf Jihad Agriculture Center Hatchery unit, were used for antibacterial investigation against six pathogenic bacteria including *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeroginosa*, *Escherichia coli*, *Salmonella* sp. *and Vibrio cholerae*. The three groups of putative bacteria (*Bacillus* sp., *Micrococcus* sp. *and Corynebacterium*sp.), collectively showed antagonistic activity against the six pathogens. *Bacillus* sp. showed greatest inhibition of pathogens both in well and disc diffusion assays. *Bacillus* sp. also inhibited all six pathogens tested by the cross-streak method. Addition of 10 ml of cell- free supernatant of *Bacillus* sp. to pathogens resulted in complete suppression within 12 h. Maximum growth and maximum zone of inhibition (16 mm), against *A. hydrophila* were accrued at pH 8. Very slow growth was observed at pH 6 and pH 9. There was no growth at pH 4 and pH 5. Maximum growth was observed at 30 °C and 37 °C with maximum production of the antibacterial component. TSB media with 1% NaCl was optimum for growth and the zone of inhibition was 15 mm. The culture reached a stationary phase at 12 h but maximum antibacterial activity was observed only at 30 h. These results suggest that *Bacillus*sp. has potential applications for control of pathogenic bacteria in aquaculture systems.

Key words: Putative bacteria, Inhibition zone,*Bacillus* sp., Antibacterial activity, *Litopenaeus vannamei*.

Pathogenic microorganisms population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and post larvae[1]. In the present day diseases have frequently affected shrimp culture all over the world. Implicated in these outbreaks were viruses, bacteria, rickettsia, mycoplasma, algae, fungi and protozoan parasites[2]. Bacteria species are microorganisms that live in the shrimp's environment, often as part of the normal microflora inhabiting the surface of their cuticle or colonizing areas of the gut or hepatopancreas[3]. For preventing and controlling from infectious microbial diseases a host of antibiotics and other chemicals have been used[2]. The use of antibiotics during shrimp cultivation has led to problems of bacterial drug resistance[4]. Now, researchers are trying to use probiotic bacteria in aquaculture to improve water quality by balancing bacterial population in water and reducing pathogenic

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bacterial load. The probiotic organisms are act as a live microbial adjunct which has a beneficial effect on the host by modifyingthe host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment[2,5,6]. The beneficial effects of probiotics have been attributed to their ability to promote the immunological and non-immunological defense barrier in the gut, normalization of increased intestinal permeability and altered gut microflora[2]. Bacteria with anti-bacterial activity have potential application as biocontrol agents and their use would be an attractive way to avoid antibiotics during shrimp culture, with no harm to the environment. Furthermore, probiotics have gained public acceptance as being more effective than administering antibiotics or chemical substances. A variety of microbes have been investigated to use as probiotics in aquaculture such as lactic acid bacteria[7,8], *Pseudomonas*[9,10,11], *Shewanella*[12,13], *Bacillus*[6,14,15,16]and commercial probiotic[17]. A common way to screen candidate probiotics is to perform *in- vitro* antagonism tests in which pathogens are exposed to the candidate probiotics or their extra cellular products in liquid mediums[18,19]or solid mediums[19,14,20,21]. Depending on the tests, candidate probiotics can be selected based on the production of inhibitory compounds[6,14]or siderophores, or on the competition for nutrients[5,22].The pre-selection of candidate probiont based on *in vitro* antagonism tests has led to the finding of effective probiotics[2,5]. The objective of this study was to find an effective probiotic of putative bacterial flora, using *in vitro* antagonism test in *Litopenaeusvannamei*.

MATERIALS AND METHODS

The putative bacterial flora of *L. vannamei* juveniles used in this study were isolated from digestive tracts of *L. vannamei*a hatchery at the Bushehr Persian Gulf Jihad Agriculture Center, located at south of Iran, 1228 km far from Tehran. These organisms were categorized as 12 groups based on the characteristics described in the Bergey's Manualof Systematic Bacteriology which included different morphological and biochemical tests[23]. At least one species of each group were identified using Biolog Diagnostic System kit and Micro station (Biolog, Inc., California, USA).Four bacteria species including *Vibrio parahaemolyticus, Aeromonashydrophila, pseudomonas aeroginosa* and *Escherichia coli* isolated from *L. vannameii* in a hatchery at the Bushehr Persian Gulf Jihad Agriculture Center, and two species of *Salmonella* sp. *and Vibrio cholerae*from Faculty Medicine, Bushehr University of Medical Sciencesused for *in- vitro* antagonism testing in this experiment as pathogens. These species were also identified in by using Biolog Diagnostic System kit and Micro station. All the cultures were maintained in Trypton Soya Agar (TSA; Difco, Colorado, USA) overlaid with sterile liquid paraffin. Before each experiment the cultures were activated in Trypton Soya Broth (TSB;Difco, Colorado, USA) and then grown on TSB for 18–24 h at 30 °C.

Study of inhibition by well diffusion, disc diffusion and cross-streak methods

Study of inhibition by well diffusion

A lawn culture of six pathogens was prepared by pouring 1 ml of a young culture (16–18 h in TSB) over the TSA medium, draining the liquid and air drying the plate in theincubator (30 °C) for 15 min. Three-millimeter-diameter wells was punched in the platesusing a sterile gel puncher. Thirty micro liters of an 18-h culture of candidates TSB was pipetted into the wells and plates were incubated for 24 h. Zone of inhibitionaround the wells was recorded[10].

Study of inhibition by disc diffusion

For study of inhibition bydisc diffusion method, the cell free supernatant of a 24-h culture of candidates in TSB was prepared by centrifuging at 10,000 × g for 10 min and filtering through a 0.22mm Millipore membrane (Millipore, USA). Discs (6.25 mm) punched from Whatman filterpaper No. 1 (USA) and sterilized in a hot air oven at 140 °C for 1 h were dipped in the cellfree supernatant and dried for 15 min in the incubator at 37 °C. The impregnated discswere placed on a lawn of six pathogens culture prepared on TSA plates and incubationContinued for 24 h at 30 °C to record the zone of inhibition around the discs[10].

Study of inhibition by cross-streak methods

For the study of inhibition by cross-streak method, an 18-h culture of candidates TSB was streaked as a 2-cm-thick band, across the diameter of the TSA plate. After incubation for 24 h at 30

°C, the growth was scraped with a sterile slide. The remaining bacteria were killed by exposure to 5 ml chloroform poured on the glass lid and left for 15 min by keeping the medium inverted over the lid. The plates were then air dried for about10 min to remove any residual chloroform and six different pathogens cultures were streaked perpendicular to candidates band using sterile glass rod dipped in an 18 h-old culture. The plates were incubated for 24 h at 30 °C. The linear zone of inhibition was recorded in each case[10].

Effect of cell free supernatant of candidates on washed cells of pathogens in sterile physiological saline

The cell free supernatant of candidates was prepared as described above. Four 250-ml flasks containing 100 ml of physiological saline were sterilized by autoclaving at 121 °C for 20 min and designated I, II, III and IV. Washed cell suspension of pathogens was added to all the beakers to get a cell density of approximately 10^7 cells ml^{-1}. 10 ml, 5 ml and 1 ml of the candidate's cell free supernatant were added to flasks I, II and III, Respectively, while beaker IV without any cell supernatant added served as control. Pathogens cells were enumerated at 0, 12 and 24 h on TSA by standard spread plate count method.

Influence of growth conditions on production of inhibitor

The influence of pH, salt, temperature and time on the antibacterial activity of candidates was studied. Sterilized TSB adjusted to pH 4, 5, 6, 7, 8 and 9 was inoculated with 0.1 ml of an 18-h old culture of candidates and incubated for 24 h at 30 °C in triplicate. Similarly, TSB with 0%, 1%, 2%, 3% and 4% sodium chloride concentration was prepared and candidates inoculated and incubated as described above. Twelve TSB test tubes, inoculated with candidates were incubated at 10, 20, 30 and 37°C for 24 h and cell free supernatant was obtained by centrifugation followed by filtration through a 0.22 mm membrane to study the antibacterial activity. From the culture incubated at 30 °C, samples were drawn at 6, 12, 24, 30, 36, 48 and 72 h to study the relation between the growth phase and production of inhibitor[24]. This relationship was analyzed by simple linear regression test[44]under using SPSS, release12, software (SPSS, Inc., USA).

RESULTS

The results of disk diffusion, well diffusion (Fig. 1) and cross-streak methods showed that three candidate probiotics (*Bacillus* sp*., Micrococcus* sp.and *Corynebacterium* sp.), collectively showed antagonistic activity against the 6 pathogens. To choose the number of candidate as a probiont, those that no inhibited even one pathogen was excluded 12 candidate probionts. *Bacillus* sp. showed good inhibition of pathogens both in well and disc diffusionassays. In well diffusion assay, the diameter of zone of inhibition was 15 mm whereas by disc diffusion assay, the zone of inhibition was 14 mm. *Bacillus* sp. also inhibited all the 6 pathogens tested by the cross-streak method.

The effect of cell-free supernatant of *Bacillus* sp., *Corynebacterium* sp. and *Micrococcus* sp. on washed cells of *Aeromonashydrophila* in sterile saline was given in Table 2. Addition of 10 ml of cell-free supernatant of *Bacillus* sp. to pathogens resulted in complete suppression of it within 12 h. Lower volumes of cell-free supernatant added to *A. hydrophila*had no inhibitory effect.

The effect of pH on the growth of *Bacillus* sp. was presented in Table 3. Growth was observed at pH 6, 7, 8 and 9 but in varying degrees. The pH 8 was found to be optimum followed by pH 7. Bacterial growth that indicated by culture media turbidity, in pH 6 and pH 9 was observed low, but there was no growth in pH 4 and pH 5. The pH 8 which showed maximum growth also showed maximum zone of inhibition (16 mm)against *A. hydrophila*. Inhibition zone observed at pH 6 and pH 9 was 8 and 9.5 mm, respectively. Table (4) showed the effect of temperature on the production of antibacterial component by *Bacillus sp*. There was low growth at 10 °C and minimal growth at 20 °C. Maximum bacterial growth was observed at 30 °C and 37 °C and production of antibacterial factor as indicated by the zones of inhibition which strongly correlated with bacteria growth (Fig. 3). Effect of NaCl concentration of the growth media on the production of antibacterial component was presented in Table 4. TSB with 1% NaCl was optimum for growth with 17 mm inhibition zone. The zone of inhibition for TSB containing 2% and 0% NaCl were 16 mm, respectively. Minimal growth was observed in TSB containing 3% NaCl

Table 1. Antagonism between candidates Probionts and pathogenic bacteria grown on TSA

Candidates	*Aeromonas hydrophila*			*Vibrio parahae molyticus*			*Escherichia coli*			*Pseudomonas aeroginosa*			*Salmonella sp*			*Vibriocholerae*		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Corynebacterium																		
Micrococcus	+	-	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-
Entrobacteriaceae																		
Aeromonas	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	+	+
Brevibacterium																		
Alcaigenes	-	-	-	+	+	+	-	-	-	-	-	-	+	-	-	+	+	
Staphylococcus																		
Acidovorax	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Vibrio	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-
Pseudomonas																		
Bacillus sp.	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+
Unidentified	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-
	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	-	-	-	-		+	-	-	-	-	+	-	+	+	-	-	+	+
	+	+	+	+	-	+	+		+	-	-	-	-	-	+	+	-	+
	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	+

1: well diffusion method; 2: Disk diffusion method; 3: Cross- streak method

Table 2. Effect of cell free supernatant of *Bacillus* sp.*, Corynebacterium*sp. and *Micrococcus* sp. on washed cells of *Aeromonashydrophila*

Sample no.	Volume of cell free supernatant (ml)	*A. hydrophila* counts ml^{-1}		
		0 h	12 h	24 h
Bacillus sp. (1)	0	3.62×10^7	5.66×10^6	4.12×10^6
Bacillus sp. (2)	1	1.53×10^7	3.60×10^7	4.31×10^7
Bacillus sp. (3)	5	2.36×10^7	3.62×10^5	1.22×10^5
Bacillus sp. (4)	10	2.86×10^7	2.31×10^3	3.2×10^1
*Corynebacterium*sp. (1)	0	3.52×10^7	6.56×10^5	3.98×10^6
Corynebacterium sp. (2)	1	1.39×10^7	3.62×10^6	1.61×10^6
*Corynebacterium*sp. (3)	5	2.16×10^7	1.13×10^6	3.62×10^5
Corynebacterium sp. (4)	10	1.52×10^7	2.82×10^5	2.98×10^3
Micrococcus sp. (1)	0	2.52×10^7	7.56×10^5	1.98×10^5
Micrococcus sp. (2)	1	1.53×10^7	3.35×10^6	2.51×10^6
Micrococcus sp. (3)	5	2.25×10^7	2.13×10^6	2.52×10^4
Micrococcus sp. (4)	10	2.29×10^7	3.62×10^5	3.92×10^4

with no antibacterial activity. The culture reached a stationary phase at 24 h but maximum antibacterial activity was observed only at 30 h (Fig. 3).

DISCUSSION

The results of disk diffusion, well diffusion and cross-streak methods showed that the *Bacillus* sp., *Micrococcus* sp. and *Corynebacterium*sp. tested in this study inhibited all the pathogenic bacteria tested.In our investigation, the isolated *Bacillus* sp. showed the best activity against shrimp pathogen. There are several *Bacillus* sp. (*B.acidophilus*, *B.subtilis*, *B.sulphureus*, *B.aerogens*, *B.radiatus*, *B.licheniformis*, etc) have been used commercially as a potential probiotic bacteria. The commercial

Table 3. Effect of pH on the growth of *Bacillus* sp. and production of antibacterial components Aeromonashydrophila

pH	Growth	Zone of inhibition (mm) at		
		12 h	24 h	48 h
4	–	0	0	0
5	–	0	0	0
6	+	0	0	8
7	+ +	0	11	12
8	+ ++	0	14	16
9	+	0	0	9.5

+: Visible growth; + +: Moderate growth; + + +: Excellent growth

Table 4. Effect of temperature and salt concentration% on the growth of *Bacillus* sp. and production of antibacterial component *Aeromonashydrophila*

Temp. of incubation C	Growth	Zone of inhibition (mm) at		
		12 h	24 h	48 h
10	+		000	
24	+		101013	
32	+ + +	141716		
Salt				
0%	+ ++		1015.516	
1%	+ ++		101617	
2%	+ +	71515		
3%	+	000		

+: Visible growth; + +: Moderate growth; + + +: Excellent growth

Table 5. Effect of incubation times on the production of antibacterial components

Time of incubation (h)	Zone of inhibition (mm)	Bacteria count(cells ml $^{-1}$)
0	0	1.16×10^5
6	0	2.16×10^6
12	13	3.21×10^8
24	15	2.18×10^9
30	16	7.35×10^9
36	16	2.16×10^9
48	16	1.96×10^9
72	16	2.11×10^8

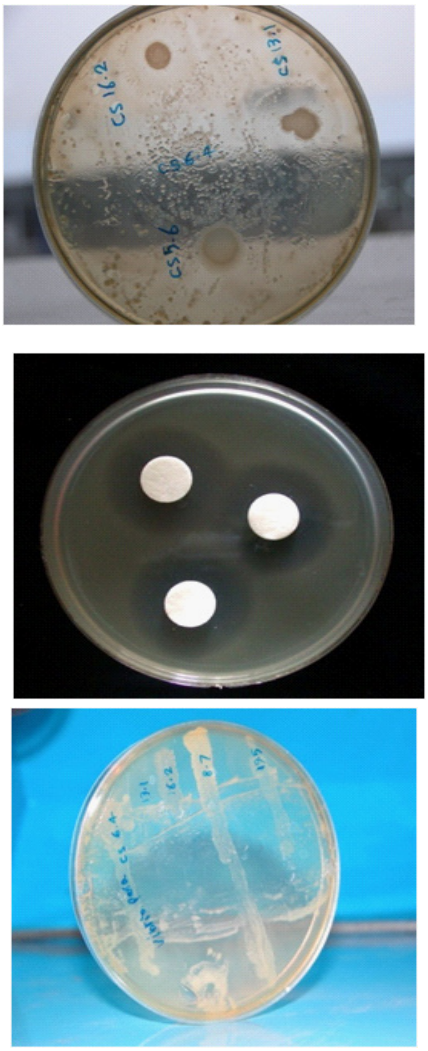

Fig. 1. diffusion method ; b: Disk diffusion method ; c: Cross- streak methoda:Well

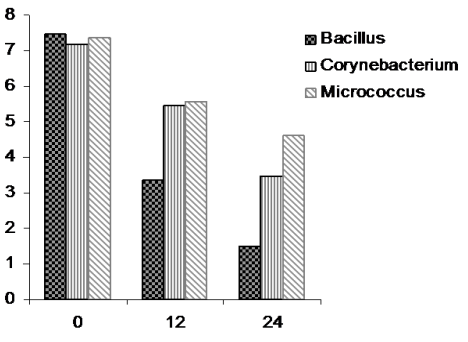

Fig. 2. Effect of 10 ml cell free supernatant of *Bacillus* sp., *Corynebacterium*sp. and *Micrococcus* sp.on viable cells count of *Aeromonashydrophila.*

probiotics were used in most of the shrimp farms. Authors have reported that a commercially prepared bacterial mixture of *Bacillus* sp. mixed into the

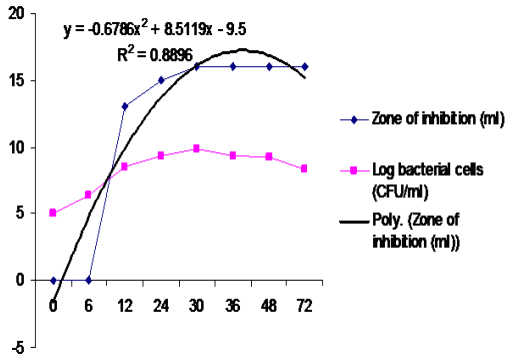

Fig. 3. Effect of incubation time on the production of antibacterial components), production of bacterial cells and correlation between inhibition zone and bacterial cells

7.3–8.0 and against *V. parahaemolyticus* at pH 6.0–8.0, whereas strain L10 showed the greatest activity against two pathogens at pH 7.3 in order to characterize the antibacterial substances against *V. harveyi*.In a research by Cheng *et al*[43]have reported that *L. vannamei* transferred from 27 °C to 34 °C (or from 28 °C to 32 °C) showed a higher susceptibility to pathogen. Numerous researchers as Balacarishnam*et al*[36]*and* Wang *et a*l[44]and Moya*et al*[45] have reported good growth and survival of *L. vannamei*in 1.7-2.3 ppt and favorable pH range from 7.6 to 8.6. Other authors,as Maeda *et al*[46] and Zokaeifar *et al*[35,47] and Rengpipat*et a49*[2]*and* Janartharam*et al*[14], documented that bacteria showingin-vitro antagonistic activity have potential application as biocontrolagents. Addition of antagonistic bacteria to the water resulted in in-vivo diseases reducing effects, and reduced numbers of pathogenic bacteria in the culture water confirmed by numerous researchers as Moriarty*et al*[49]and Gram *et al*[19] andKeysamei*et al*[24,34]. In conclusion, it can be stated that the Bacillus sp. strain in this experiment has properties of a biocontrol agent with potential to be used in prawn hatcheries and farms. Proiotic ability of this species including maximum growth and production of the antibacterial components were observed under conditions pH 8, 30 oC, 1% NaCl and 30 h rearing water increased survival and production of channel catfish[16]. Antagonistic activity of *Bacillus* sp. against pathogens such as *Salmonella* sp.,*Vibrio* sp.*, Aeromonas*sp. and *Escherichia coli* has been reported in the literature byJanartharam*et al*[14]and keysamei*et al*[5]and Zokaeifar*et al*[6].Keysamei*et al*. isolated a strain of *Bacillus* sp. that was antagonistic to most of the bacterial isolates from *Macrobrachium rosenbergii*[25]. Results of this study including production a wide zone of inhibition against pathogenic bacteria can be depended to production of some extra cellular anti-bacterial components by *B. subtilis*. This is confirmed by reports which show that *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds[26,27,28]. It produces novel antibiotics such as difficidin and oxydifficidin that have activity against a wide spectrum of aerobic and anaerobic bacteria as well as more common antibiotics such as bacitracin, bacillin, and bacillomycin B[29,30].The appearance of the antibacterial activity in the medium when the cells reach stationary phase of growth and maximum activity at stationary phase (Fig. 3), suggests that the antibacterial factor is a secondary metabolite.

These results are very important since *A. hydrophila* and *Vibrio* sp. are great problems in prawn hatcheries and confirmed by Austin*et al*[31] and Hoa *et al*[32] and Keysamei*et al*[33,34]and Soundarapandian *et al*[1]and Ma.del*et al*[3]andZokaeifar*et al*[35]. On the other hand, the maintenance of good water quality is essential for optimum growth and survival of shrimp. Good water quality characterized by adequate temperature, pH and salinity[36].The most important environment including temperature, salinity and pH, which significantly influence shrimps growth[37]. In the present studythe maximum production of antibacterial factor was observed at pH 8, 30 °C and low salinity (1 gL^{-1}) which was also the optimum pH, temperature and salinity for the growth of the shrimp byJesus *et al*[38]and New and Singholka[39]and Kumla*et al*[40]andTian*etal*[41] and Zhang*et al*[42]. Similar to our study, Zokaeifar*et al*[6] were tested *B. subtilis* for antibacterial activity under different physical conditions, salinity, pH and temperature using the agar well diffusion assay. Strains showed the highest level of antibacterial activity against two pathogens at 30 °C and 1.0% NaCl. Under the pH conditions, strain G1 showed the greatest activity against *V. harveyi* at pH

of incubation respectively.The result of the present investigation thus evinced that the isolated bacteria inhibited the pathogens in vitro. It appears to be an effective way of controlling pathogen, which could substitute the negative impacts of the use of antibiotics in aquaculture.

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