J. Agric. Sci. Mansoura Univ., 29 (12): 6853 - 6871, 2004

USE OF *Bacillus subtilis* IN MICROPARTICULATE DIETS FOR PRODUCING BIOSECURE *Peneaus japonicus* POSTLARVAE

El-Dakar, A.Y. [1] and Y.M. Gohar[2]

1- Fish Resources and Aquaculture Department, Faculty of Environmental Agric. Sciences, Suez Canal Univ., El-Arish. Egypt.

2- Microbiology Division, Fac. of Science, Alex. Univ., Alexandria, Egypt

ABSTRACT

Two separate feeding trails were carried out to study the effect of *Bacillus subtilis* in probiotic microparticulate diets on survival, growth and pathogen resistance of *Penaeus japonicus* larvae and postlarvae. The first one was done on an experimental scale while the second was done on a large scale as an application trail. Probiotic diet was formulated by adding 500 mg of *Bacillus subtilis* ($7x10^7$ cell/g) per 100 g basal diet that containing 53% protein with particles size of 350–800 µm. In the experimental trail, 1000 postlarvae of *P. japonicus* at substage of PL_1 were randomly distributed, stocked into six circular-conical bottom fiberglass tanks; each one had a capacity of 100-L. In the application trail, two 3.6 m^3 U-shape fiberglass tanks were used to stock 180,000 hatched nauplii of *P. japonicus* in each. Survival of postlarvae fed probiotic diet was higher than those fed the basal diet. Enhanced growth was generally obtained in postlarvae fed probiotic diet compared with the basal diet in either experimental or application trails. Challenge of bacteria to pathogen, as a universal probiotic bacterium, was studied by evaluating its action against three different aquaculture bacterial pathogens, namely, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Vibrio proteolyticus*. A considerable change in the intra and extra-cellular proteins profiles of the three bacterial pathogens were observed when electrophoresed *via* SDS-PAGE techniques after mixing with *B. subtilis*. The proteolytic activity of the bacterial pathogens exhibited a sharp decrease when subjected to *B. subtilis* extra-cellular products. In addition, it was noted a positive effect of the extra-cellular products of *B. subtilis* against the pathogens and on reducing the antibiotic susceptibility when presented in culture water or in feed of shrimp.

Keywords: Probiotic, Shrimp, Biosecure, Nutrition, Postlarvae, *Aeromonas, Edwardsiella, Vibrio*

INTRODUCTION

Bacterial and viral diseases are known to be the major constraint in the further progress of semi-intensive and intensive shrimp culture throughout the world. In Taiwan for example, shrimp production in the years 1987-1988 decreased by 60% (Wyban *et al.*, 1992) and by 65% in Ecuador in the year 2000 (Rosenberry, 1998), due to massive mortalities caused by pathogenic microorganisms. Egypt faces a similar situation in shrimp culture as a consequence of the white spot syndrome virus (WSSV) and many farms are stopped. Pathogenic bacteria, especially *Aeromonas*, *Edwardsiella* and *Vibrio* spp (Baticados *et al.* 1990) have been involved in this crisis and nutrition may play a vital role in this context. The high density of animals in hatchery tanks and ponds is conducive to the spread of pathogens, and the aquatic

environment, with regular applications of protein-rich feed, is ideal for culturing bacteria.

Proper nutrition has long been recognized as a critical factor in promoting growth and sustaining health of shrimp. Prepared diets not only provide the essential nutrients that are required for normal physiological function but also may serve as the medium to other components that may affect the health of shrimp (Gatlin, 2002). Although the concept of functional feeds is novel to the aquaculture industry, it represents an emerging new paradigm to develop diets that extend beyond satisfying basic nutritional requirements of the cultured organisms (Li and Galtin, 2004). Research on optimization of diets to enhance health is still in its infancy. Probiotic live microbes that may serve as dietary supplements to improve the intestinal microbial balance have received some attention in aquaculture (Irianto and Austin, 2002 and Gullian *et al.*, 2004). The search of probiotic for aquaculture is increasing with the demand for environmental-friendly aquaculture to produce biosecure animals. Bacteria that have been used successfully as probiotic belong to genus *Vibrio* (Griffith, 1995) and *Bacillus* spp (Moriarty, 1998). Most researchers have isolated these probiotic strains from shrimp culture water (Tanasomwang *et al.*, 1998) or from the intestine of different penaeid species (Rengpipat *et al.*, 2000).

The use of probiotic for terrestrial animals is well-developed and is attracting more attention, particularly as the use of antibiotics in farm animals has come under increasing pressure (Healey, 2004). Probiotics are also of great benefit in aquaculture and allow the use of chemicals to be greatly reduced or eliminated. In addition, probiotic may serve to produce biosecure shrimp in hatcheries and growout ponds. Therefore, shrimp farms should be depend on postlarvae that have disease-resistance and fast growth (Wyban, 2000). Production of biosecure postlarvae, either specific pathogen free (SPF) or highly resistant for pathogen (HRP) may be achieved by culture methods, nutrition or a combination of them. Biosecure production systems depend upon presence of bacteria especially heterotrophic bacteria including *Bacillus* (Irianto and Austin, 2002). Several commercial probiotics are currently marketed for use to treat the culture water prior to and during stocking and cultivation of fish and shrimp. Most of these preparations are stabilized forms of various strains of *Bacillus subtilis*. The present study therefore aimed to use *B. subtilis* in microparticulate diets for larvae of *Penaeus japonicus* to enhance growth and survival and to produce biosecure postlarvae.

MATERIALS AND METHODS

Two larval feeding trails, one on an experimental scale and its application on a commercial scale are carried out at Mariculture Research Center, Faculty of Environmental Agricultural Sciences, Suez Canal University, El-Arish, Egypt to study the effect of usage probiotic on shrimp, *P. japonicus*, larval feeding.

Experimental animals and its rearing

Gravid females of shrimp were collected from the Mediterranean Sea, Abou-Kir, Alexandria, Egypt. They were selected from live shrimp caught by fishermen using bottom gill net. The selected animals were transported in polyethylene bags filled at a ratio of 1:3 water: oxygen placed in Sterofom box. Upon arrival, gravid females of shrimp were stocked in 1 m^3 circular-conical bottom fiberglass tank to 48 hrs for spawning and hatching. Hatched nauplii were harvested and stocked into larval rearing tanks (3.6 m^3 U-shape fiberglass tank). The tanks were provided with filtered seawater and fine aeration. Diatoms of *Skletonema costatium* were used for larval feeding during their early stages as described by El-Dakar (2001). Starter of algae was obtained from SEAFDEC, Iloilo, Philippine. Algae were cultured in seawater supplemented with a culture medium of Walne and maintained in a chamber under controlled conditions (20±2°C, 20 ppt salinity, and illumination of 1000 lux for 12h /12h, L/D daily) as described by Lavens and Sorgeloos (1996). Feeding with *Artemia* nauplii was started at mysis stage (M_1). The source of *Artemia* cysts was Great Salt Lake (GSL), Salt Creak Co., USA. The cysts were treated with sodium hypochlorite for decapsulation and incubated in seawater to hatch as described by Lavens and Sorgeloos (1996). Feeding schedule used in this study is given in Table (1). The animals were reared at ambient salinity (37 ppt), temperature (30±2°C), pH (8.7±0.2), DO (8±2 ppm) and natural light.

Experimental design

Two separate feeding trails were carried out in this study. First one was done on an experimental scale while the second was done on a large scale as an application trail.

1- The experimental trail

Postlarvae of *P. japonicus* at substage of PL_1 were randomly distributed and stocked into six circular-conical bottom fiberglass tanks, each one had a capacity of 100-L. One thousand of PL was stoked into each tank. Postlarvae were fed basal and probiotic diets with particles size of 500-800 µm, in triplicate. Shrimp larvae were fed on a constant amount of feed (3 g/tank daily at six meals) for 21 days.

2- The application trail

Two 3.6 m^3 U-shape fiberglass tanks were used to stock 180,000 hatched nauplii of *P. japonicus* in each. Shrimp larvae were fed *Skeletonema costatum* at N_6 (El-Dakar, 2001) and *Artemia* nauplii at M_1 (Lavens and Sorgeloos, 1996). Artificial feeding on the basal and probiotic diets was started at M_1 with microbinding diets (Agar-MBD). The particle size of microparticulate diets was 350 µm and increased with larval stage development until 800 µm.

All tanks in both trails were provided with filtered seawater. Water quality in the tanks was maintained through partial change of third water daily by new filtered seawater. Dissolved oxygen levels were maintained by an air blower and air stones.

Bacterial mixture preparation

Bacillus subtilis strain was obtained in a powder form (Versuchsprapart Co, Germany; BS03; $7x10^{5}$). A bacterial mixture was prepared by mixing 10 mg of *Bacillus* powder with 990 mg of wheat flour to give approximately $7x10^{7}$ cell per gram.

Table (1). A feeding schedule of larvae used in the application trail.

Stage	Algae X 10^3 cell/ml	*Artemia*	Agar-MBD g/ tank
N_6/Z_1	100	-	-
Z_1- Z_3	120		
M_1	100	-	-
M_2	80	0.5	-
M_3	60	1.0	-
PI_1	60	1.5	-
PI_2	40	2.0	2
PI_3	20	3.0	4
PI_4	20	4.0	6
PI_5	10	6.0	8
PI_6	10	8.0	10
PI_7	5	8.0	12
PI_8	5	6.0	12
PI_9	-	6.0	14
PI_{10}	-	4.0	14
PI_{11}	-	4.0	20
PI_{12-15}	-	2.0	25

Experimental diets

The basal diet used in this study utilized fish meal, shrimp meal, squid meal and soybean meal as the protein sources to formulate 53% protein, 13% ether extract and 518 kcal GE100 g diet. All ingredients used in this study were collected from locally available feedstuffs that are used in practical diets of shrimp. Chemical analysis of feedstuff is given in Table (2).

Table 2. Chemical composition of ingredients used in the experimental diets.

Ingredient	DM	% on DM basis				
	%	CP	EE	CF	NFE	Ash
Fishmeal[1]	93.86	65.11	12.21	1.05	7.51	14.12
Shrimp meal[2]	89.89	43.91	27.31	8.33	13.74	6.71
Squid meal[2]	85.05	36.14	30.47	4.61	25.48	3.30
Soybean meal[3]	91.18	42.66	16.88	5.43	28.54	6.54
Wheat flour[4]	87.61	10.81	5.36	6.30	75.33	2.20

1. Herring fish meal, Revisen, Co, Denmark.
2. Home made by drying of fresh product.
3. Kafer El-Zayyate Extracted Oils Co., Kafer El-Zayyte, Egypt.
4. East Delta milling Co., El-Arish, North Sinai, Egypt.

Probiotic diet was formulated by adding 500 mg of bacterial mixture (70x 10^7 cell *B. subtilis* /g) to 100-g basal diet. Ingredient composition of the basal and probiotic diets and their chemical composition are given in Table (3). A microparticulate diet technique was used to prepare the two experimental diets. All ingredients were powdered into fine particles prior to the processing of diets as Agar-MBD according to Teshema *et al.* (1982). The dried diets were sieved to obtain the particles with the proper sizes (350-800µm).

Table (3). Ingredient composition (%) and proximate analysis (%) of the diets

Ingredient	Basal diet	Probiotic diet
Fishmeal, Herring meal	60	60
Shrimp meal	5	5
Squid meal	5	5
Soybean meal	12	12
Wheat flour	9	8.50
Cod liver oil	2.5	2.5
Sunflower oil	2.5	2.5
Vitamin premix1	1	1
Mineral premix2	2	2
Cholesterol	0.5	0.5
Vitamin C	0.5	0.5
Probiotic powder	0	0.05
Proximate analysis		
Dry matter %	98.1	98.11
Crude protein	53.24	53.09
Ether extract	13.72	13.52
Crude fiber	2.6	2.7
Nitrogen free extract	10.81	10.78
Ash	19.63	19.91
Gross energy[3] kcal/100g	518	474

1. **Vitamin mixture, each 100 g contain 960000 IU, 160000 IU, 0.8 g, 80 mg, 0.32 g, 0.12 g, 0.8 g,, 0.8 mg 1.6 g, 80 mg, 4 mg, 40 g. of vitamin A, D3, E, K, B1, B2, B6, Pantothenic acid, B12, Niacin, Folic acid Biotin, Choline chloride, respectively.**
2. **Mineral mixture, each 100 g contain 12.75, 72.85, 0.55, 0.25, 0.02, 5, 2.5, 0.08, 0.05, 0.01 and 6 mg of $MgSO_4.7H_2O$, $CaHPO_4$. $2H_2O$, $ZnSO_4$. $7H_2O$, $MnSO_4$. $4H_2O$, $Cal_2.O_6$. $6H_2O$, KCl, $FeSO_4$. $7H_2O$, $CuSO_4$. $5H_2O$, $CoSO_4$. $7H_2O$, CrC_3. $6H_2O$ and NaCl, respectively.**
3. **According to NRC (1993).**

Protein profile of the intra and extra-cellular

All bacteria were cultured in sheep-blood medium (supplemented with 0.5% sodium thioglycolate for the three pathogens) and incubated overnight at 37°C with moderate shaking. Cells were sedimented at 3000 *g* for 15 min and then washed in phosphate buffered saline (pH 7.2), resuspended in 15% glycerol, 1% sodium dodecyl sulfate (SDS) and 0.1 M Tris/HCl, pH 6.8, and denatured by treatment at 100°C for 20 min. Nonsolubilized material was removed by centrifugation at 3000 g for 15 min and the resulting supernatant

was diluted 1:1 with 20% glycerol, 10% 2-mercaptoethanol, 4% SDS and 0.125 M Tris/HCl, pH 6.8 (Whittington *et al.*, 1987). The protein concentration of the supernatant was adjusted to 0.75 µg/µl with the same buffer (Lowry *et al.*, 1951). After incubation for a further 2 min at 100°C, the samples were stored at -20°C for electrophoresis. Then 20 µl of the sample was loaded on to the gel. SDS-PAGE was carried out at constant current (300 V). The resolving gel contained 12% acrylamide/bis acrylamide in a ratio of 29:1 with a stacking gel of 4.75% with respect to total acrylamide (Whittington *et al.*, 1987). Other running conditions and buffers were used as described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Blue (0.025% Coomassie Blue R-250, 40% methanol, 7% acetic acid) for 3 h. The gel was kept in destaining solution I (50% methanol, 10% acetic acid) for 1 h and then transferred to destaining solution II (7% acetic acid, 5% methanol) (Hoefer Scientific Instruments, 1992-1993. SDS Gels, San Francisco, USA.).

Proteolytic activity of the extra-cellular proteins

This was done as the SDS-PAGE technique but adding 4% casein (as a substrate) before polymerization of the gel skipping the step of adding SDS. After electrophoresis, the gel plate was incubated for 3 hrs to activate the proteolysis process, stained with Coomassie Blue staining solution for 3 hrs and finally destained as previously described to visualize the hydrolysis areas of casein that correspond the presence of proteolytic enzymes.

Challenge of bacteria (minimum inhibitory concentrations, MICs)

Bacterial strains were tested for susceptibility to a panel of six antimicrobial agents: Bacitracin, Erythromycin, Gentamicin, Oxytetracycline (terramycin), Penicillin G and Streptomycin. The test was performed by use of a broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1990). One ml heavy suspension of *B. subtilis* (10^8 CFU/ml), grown over night in LB medium at 30°C, was used to inoculate 25 ml aliqots with or without *B. subtilis* in 100-ml conical flasks, incubated with shaking (120 rpm) for 24 hrs at 30°C. Culture filtrates containing the extra-cellular products were aseptically separated by centrifugation at 3000 *g* for 30 min and kept at -20°C until the time of use. All culture media were used for production of the extra-cellular products for MIC determination in presence of the extra-cellular products of *B. subtilis* (the probiotic), the previously mentioned micro-plate method under the standards of NCCLS (1990) was modified by using the four mentions media.

Chemicals, media and strains

Sodium thioglycolate,Tris/HCl, acrylamide, SDS, Coomassie Blue, protein markers for SDS-PAGE, 2-mercaptoethanol and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Culture media were from LAB M (http://www.lab-m.com/culture.htm). Other regular chemicals were all analytical grade. *Bacillus subtilis, Aeromonas hydrophila, Edwardsiella tarda* and *Vibrio proteolyticus* used in this investigation were biochemically identified according to Probabilistic Identification of Bacteria for

Windows. Version 1.9.2. (Bryant, 2002). Proximate analysis of feed was carried out by standard methods for crude protein, crude fat, crude fiber and ash (AOAC, 1990). Nitrogen free extract was calculated by difference.

Statistical analysis

Data of the 1 st experiment were statistically analyzed as a complete randomized design in triplicates according to Snedecor and Cochran (1982). A significant level of $P<0.05$ was used.

RESULTS

The experimental trail

At the end of the experimental trail, survival of postlarvae fed probiotic diet was significantly ($P<0.05$) higher than shrimp fed the basal diet (Fig. 1). The same trend was found for final weight and weight gain. Consequently, enhanced growth was generally obtained in postlarvae fed probiotic diet compared with the basal diet (Fig. 2). *Bacillus* sp improved growth by 53.3% of the control group (Fig. 2). Initial larval length and carapace of shrimp were similar, but at the end of the experiment, final and gain of total length of shrimp fed *Bacillus* were higher than those fed the basal diet (Fig. 3).

The application trail

On the large-scale production, survival of shrimp during the larval development from N6 to PL30 is given in Fig. (4). It is clear that, most of larvae in both treatments before use of inert diet gave similar survival rate at early stages (until ZIII). But survival was higher with starting feeding with probiotic diet than those fed the basal diet at mysis and PL stages. It recorded 119,000 *vs* 77,000 PL, represented 66 and 48% for PL fed probiotic and basal diet, respectively (Fig. 5). Relative growth of shrimp larvae fed the probiotic diet tended to be higher than those fed the basal diet (Fig. 5).

Challenge of *B. subtilis*

SDS-PAGE protein (denatured) profile obtained from the whole cell extract (intracellular soluble proteins) of *A.hydrophila, E. tarda* and *V.proteolyticus* when grown with or without *B. subtilis* (Fig. 6) showed the response of these profiles to the action caused by the extra-cellular secretions of the probiotic *B. subtilis*. The same response was observed with the active extra-cellular products (proteins and non-proteins) in the culture media (Fig. 7). Both figures showed the reflection of *B. subtilis* action that can simply lead to a complete disappearance or appearance of some protein bands and in other cases the concentration of protein bands differ much due to the treatments. The action of the presence of *B. subtilis* on the proteolytic activity of the three pathogens was visualized *via* the native gel electrophoresis (Fig. 8) reflecting a dramatic decrease in both quantity and quality of the residual proteolytic enzymes secreted by these pathogens. Such an action will decrease the virulence of the pathogens and limits to a great extent the muscles and body damage of the growing shrimp.

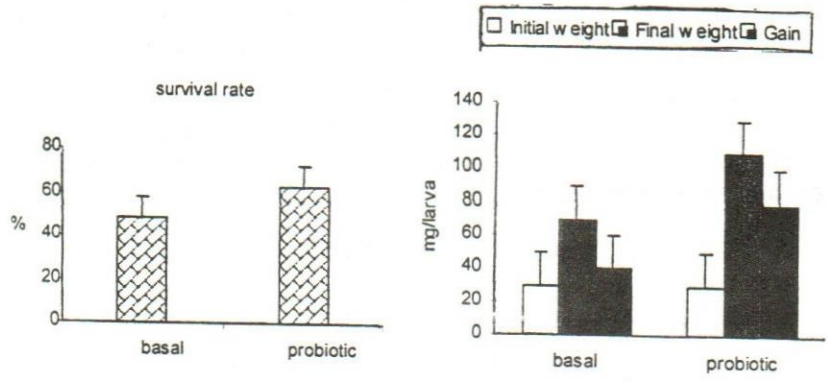

Fig. 1 Survival rate and initial, final and gain of weight of *P. japonicus* fed probiotic and the experimental diets. Values mean (n=3).

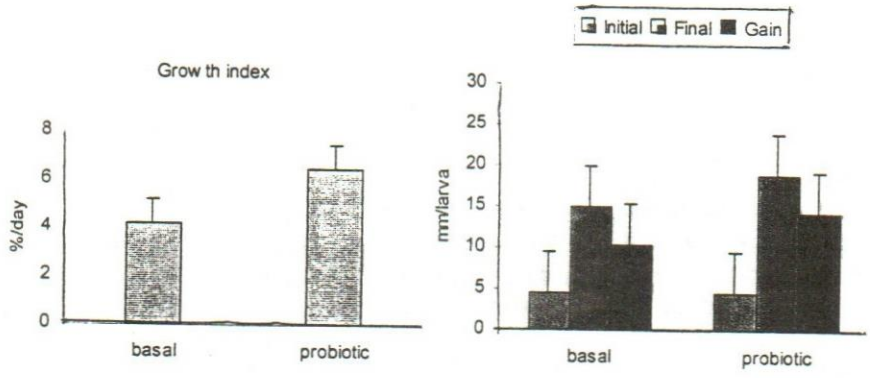

Fig. 2. Growth index and initial, final and gain of weight of postlarvae, *P. japonicus* fed probiotic and the experimental diets. Values mean (n=3).

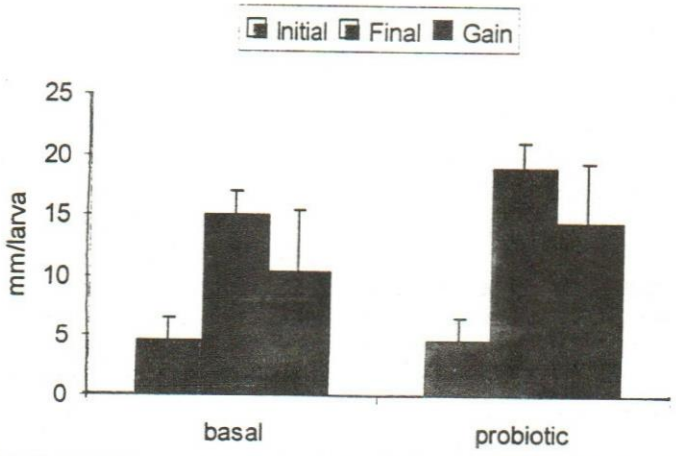

Fig. 3. Initial, final and gain of carapace length of postlarvae, *P. japonicus* fed probiotic and the experimental diets. Values mean (n=3).

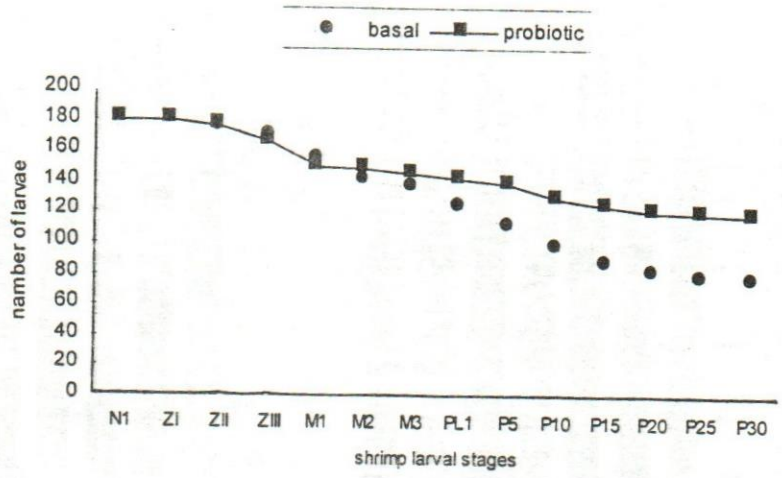

Fig. 4. Survival shrimp postlarvae fed probiotic diet in the application trail.

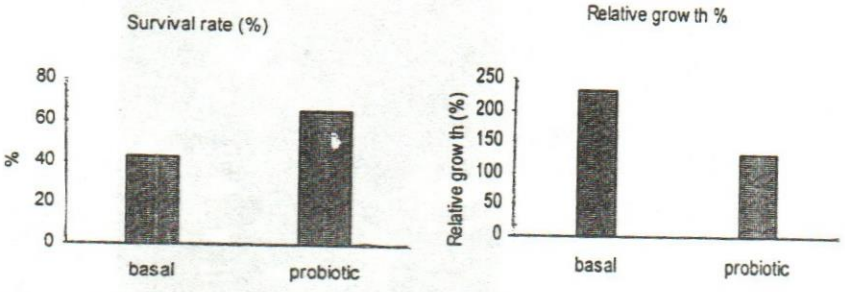

Fig. 5. Survival rate and relative growth of shrimp postlarvae fed probiotic diet in the application trail.

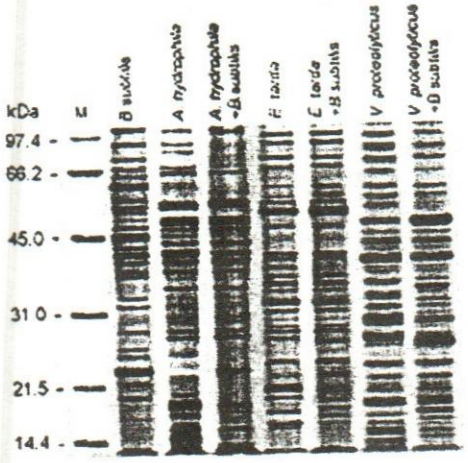

Fig 6. Protein profiles of intracellular soluble proteins (whole-cell extracts) of *A. hydrophila*, *E. tarda* and *V. proteolyticus*, with or without *B. subtilis* separated by SDS-PAGE.

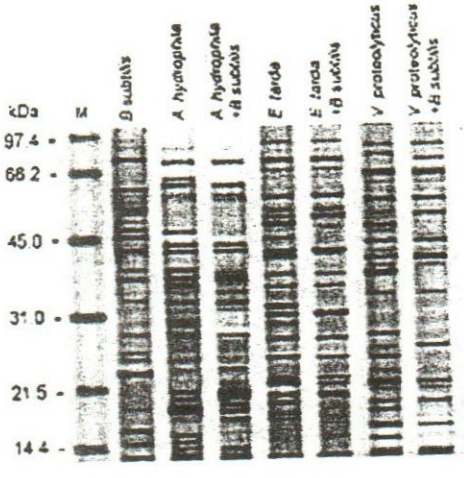

Fig 7. Protein profiles of the extra-cellular proteins of *A. hydrophila*, *E. tarda* and *V. proteolyticus*, grown alone or in presence of *B. subtilis* separated by SDS-PAGE.

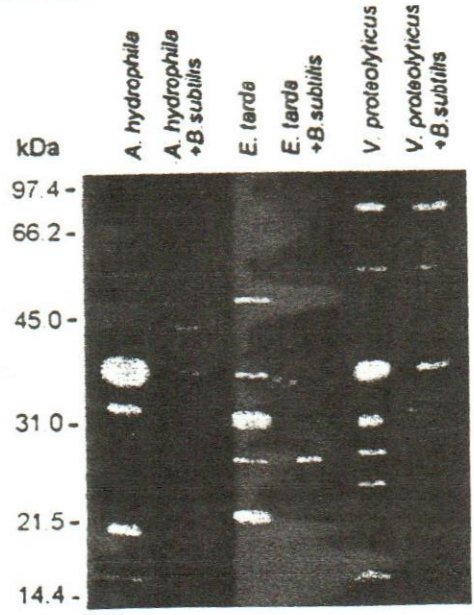

Fig 8 . Native gel of the extra cellular proteins of *A. hydrophila*, *E. tarda* and *V. proteolyticus* , grown with or without *B. subtilis* showing the proteolytic activity.

Addition of *B. subtilis* cells to cultures containing the three pathogens resulted in a considerable increase in the susceptibility of the pathogen compared to the control treatment. Order of reduction in susceptibility was as follows: *V. proteolyticus* (66%) > *A. hydrophila* (45%) > *E. tardae* (32%) as shown in Fig. (9).

The presence of a relation between *B. subtilis* extra-cellular competing products and the susceptibility of the tested pathogens to the representative antibiotics. This may lead to minimize usage of antibiotics in postlarvae culture or to prohibit it completely. Presence of *B. subtilis* cells, with its extra-cellular secretions, exhibited a noticeable synergistic action on the average (mean of MIC values for the three pathogens under all culture conditions) activity of the antibiotics used in this investigation. The average potency of the six antibiotics against the three bacterial pathogens were as follows (MIC expressed as µg/ml): streptomycin > gentamicin > bacitracin >oxytetracycline > ampicillin > erythromycin (Table 4). Variability in over all response (indicated by standard deviation value) of average antibiotic susceptibility are given too. The least SD value was observed for *A. hydrophila* followed by *E. Tardae* then *V. proteolyticus* (Fig. 10). The low SD value for *A. hyfrophila* data group may indicate that the response of this pathogen to different antibiotics is not highly affected by the changes in the culture conditions. On the other hand, *V. proteolyticus* is considerable to be much affected by the constitution of the culture medium.

Table 4. Minimum inhibitory concentration (MIC,mcg/ml) for some representative antibiotics with *A. hydrophila*, *E. tarda* and *V. proteolyticus* grown in different culture media in presence of the extra-cellular product of *B. subtilis*.

	A. hydrophila				*E. tarda*				*V. proteolyticus*				
Antibiotic	Control	SCW	SW-LB	SW-SH	Control	SCW	SW-LB	SW-SH	Control	SCW	SW-LB	SW-SH	Average MIC for each antibiotic
Ampicillin	32	4	8	4	16	4	16	16	64	16	8	16	17
Bacitracin	8	8	8	8	32	4	16	16	32	32	16	16	16
Erythromycin	16	4	16	8	16	4	16	16	64	8	4	4	15
Gentamicin	4	4	4	2	8	8	9	4	32	16	8	8	9
Oxytetracycline	16	8	16	8	16	4	9	32	16	32	8	4	14
Streptomycin	8	16	8	4	8	4	2	16	4	16	2	2	8
Average of treatment	14	7	10	6	16	5	11	17	35	20	8	8	

Control = Raw sea water (Mediterranean).
SCW = Shrimp culture water
SW-LB = Sea water-LB medium (LB medium prepared with sea water instead of distilled water.
SW+SH = Sea water-Shrimp homogenate (1% shrimp flesh). Ten g fresh shrimp flesh were mixed with 90 ml cold sea water, homogenized in a blender, diluted with sea water to a final volume of 1 L.

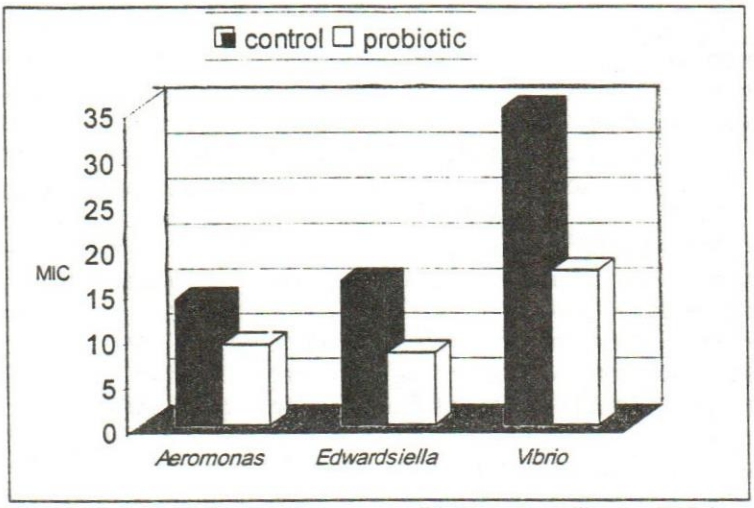

Fig. 9. Effect of *B. subtilis* challenge on average antibiotic susceptibility (as MIC in µg/ml) for different pathogen microbes.

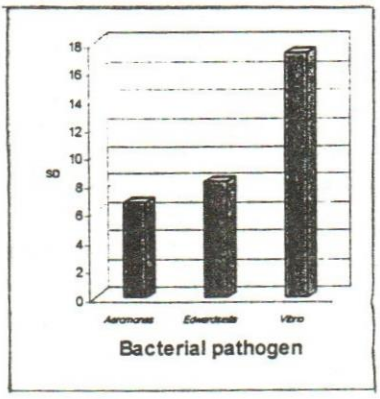

Fig. 10. Standard Deviation (SD) for the over all response of different bacterial pathogens to antibiotics and different culture media (based on data given in Table 1).

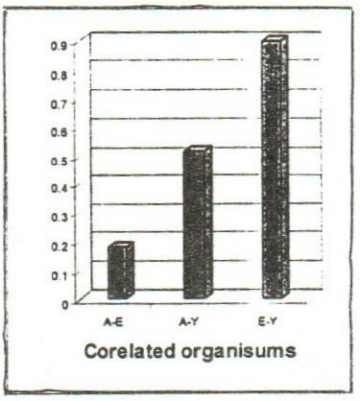

Fig. 11. Correlation coefficients among different bacterial pathogens to antibiotics and different culture media (based on data in Table1). A=*Aeromonas*, E=*Edwardsiella*, and V=*Vibrio*..

Correlation analysis of the response to different antibiotics in different culture media between different pairs of pathogens are given in Fig. (11). Correlation coefficient between *Aeromonas* and *Vibrio* (E-V) was relatively high but it was low between *Edwardsiella* and *Vibrio* (E-V). This may give an idea about the expected similarity in response of a pathogen when the response of another pathogen is known.

DISCUSSION

Microencapsulate diets used in the present study is useful to allow the manufacture of stable particles that may potentially prevent excessive nutrient leaching as well as the subsequent water pollution (Wilson, 1989 and El-Dakar *et al.*, 1999). In addition, agar micro-binding diets might be good to control the appropriate size required for shrimp at the weaning stage (El-Dakar *et al.*, 1999). Although, limited success has been achieved when inert feed is produced as first food for species such as sea bass (Cahu *et al.*, 1998), red sea bream (Takeuchi, 2001) and other (Southgate and Partridge, 1998). Weaning of shrimp larvae to inert diets at a later stage of development, however, is easily achieved by co-feeding inert feed with live food e.g. *Artemia* (El-Dakar *et al.*, 1999; Kolkovski, 2001 and Takeuchi, 2001). The present study confirms the above findings; probiotic diet including *Bacillus* gave higher survival rate and better performance of *P. japonicus* postlarvae. *Bacillus* may serve in this case as a co-feeding of inert feed and may help to maximize the microparticulate diets efficiency through stimulating digestive tract. Co-feeding not only stimulates the ingestion of feed particles, but also, promotes digestion and assimilation of micro-diets by larvae (Koven *et al.*, 1998).

The level of survival of *P. japonicus* postlarvae was high in shrimp fed the probiotic diet in both trails. While survival was declined in the control shrimp fed the basal diet. These results are consistent with those obtained by Moriarty (1998) who noted an increase of prawn survival in ponds where some strains of *Bacillus* spp were introduced. It is possible that the probiotic supplemented diet provided more optimal nutrition than the basal diet. Final body weight, weight gain and growth index of shrimp fed probiotic diet were significantly ($P<0.05$) higher than those fed the basal diet. Similar results were obtainedfor PL_{30} of *P. monodon* (Rengpipat *et al.*, 1998) and for juvenile *P. monodon* (Rengpipat *et al.*, 2000) using *Bacillus* S11 as probiotic in the feed. Recently, Gullian *et al.* (2004) reported that a significant growth increase was observed in the shrimp inoculated with *Bacillus* P64, *Vibrio* P62 and *V. alginolyticus* (LLi) compared with the control. These results may be attributed to the *Bacillus* effect in improving digestive activity by synthesis of vitamins and cofactors or enzymatic improvement (Gatesoupe, 1999). These probiotic effects could be the cause of the increased weight, digestion improvement or nutrient absorption. This phenomenon operates by substitution of depressive microbial agents which hinder growth (Gullian *et al.*, 2004). Also, the growth promotive effect is conditioned to ambient factors; therefore; the results are subjected to a high degree of variability.

Consequently, the probiotic used as growth stimulant can yield different results under different culture conditions.

Several mechanisms have been suggested as modes of action for probiotic bacteria. The competitive exclusion mechanism, based on the substitution of pathogen by the beneficial population, has been considered to be important by many authors (Moriarty, 1998; Gatesoupe, 1999 and Li and Galtin, 2004). Also, stimulation of the immune system using probiotic strains has been reported by Rengpipat *et al.* (2000). Furthermore, superiority of *Bacillus* in survival, growth and health status may be due to its biocontrol or bacterial antagonism effect and its production of antimicrobial agents such as antibiotic, and antimicrobial substances. Sugita *et al.* (1998) isolated a strain of *Bacillus* sp that was antagonistic to 63% of the isolates from fish intestine.

SDS-PAGE protein profiles of the intra and extra-cellular proteins of the three shrimp pathogens, *A. hydrophila*, *E. tarda*, and *V. proteolyticus* due to the presence of *B. subtilis* cells exhibited noticeable change in both quality and quantity of the protein bands. Some of these bands may be corresponding to some enzymes and some others to some peptide antibiotic or antagonistic factors. Adherence of *Aeromonas* strains in different marine (Millership and Want, 1993) hosts was found to be dependent on membrane proteins of the organs as well as the proteins secreted by these pathogens. This process was also found to be dependent on the environmental conditions including media conditions and temperature (Millership and Want, 1993). An extra-cellular protease (with elastolytic activity) isolated from *A. hydrophila* AG2 hydrolyzed casein and elastin. This protease was demonstrated to have an important role in its pathogenesis (Casco *et al.* 2000). The pathogenicity of *A. hydrophila* (and related aeromonas) has been attributed to several characterized extra-cellular enzymes including hemolysins, enterotoxins, and proteases (Janda, 1985).

Some *Bacillus* escaped to the cultural environment via experimental feed producing its secretions (extra-cellular proteins). The *Bacillus* secretions resulted in a positive effect against the test pathogenic microbes. The native gel, for the proteolytic activity changes of the three pathogens in the presence of *B. subtilis* cells and its extra-cellular products revealed the active role of *Bacillus* secretions on the overall proteolytic activity of each of the pathogens. This may reflect lights on the antiproteolytic activity of the enzymes produced by these pathogens. Moreover, the reduction of the number of proteolytic bands may be attributed to the antagonistic capabilities of some *B. subtilis* products on the mechanisms of production and/or the activity of their virulent proteolysis. The proteolytic activity (against Hide Powder Azure) and haemolytic activity (against horse erythrocytes) were confirmed in cell-free filtrates from four strains of *Aeromonas hydrophila* which were grown under a range of commercially relevant modified atmospheres (Joanne *et al.*, 1989). Protein and enzymatic activity of marine pathogens were found to be directly or indirectly affected by the culture conditions. *Vibrio anguillarum* serotype O2 strains express a 40-kDa outer membrane porin protein that increased by growth in CM9 medium containing 5 to 10% sucrose or 0.1 to 0.5 M NaCl at 15°C. In contrast, the levels of the protein were significantly reduced when

cells were grown at 37°C, and a novel 60-kDa protein was also observed (Davey *et al.*, 1998).

Alive bacteria and exo-cellular protein of two *Vibrio* marine pathogens (*V. penaeicida* and *V. nigripulchritudo*) exhibited significantly high moralities in blue shrimp species *Litopenaeus stylirostris* (Aguirre-Guzman *et al.*, 2003). *Bacillus subtilis* is currently used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders (mostly as a direct result of antibiotic treatment). Ingestion of significant quantities of *B. subtilis* is thought to restore the normal microbial flora following extensive antibiotic use or illness (Green *et al.* 1999). In addition, Moriarty (1998) stated that the use of *Bacillus* has been promoted and accepted within the industry due to it has not been associated with aquatic organism pathologies. The present study confirms the above findings. Gatesoupe (1999) reported that probiotic treatment decreased the proportion of pathogenic *Vibrio* spp in the sediments and to a lesser extent, in the water.

Due to the reflection of antibiotic resistance problem in aquaculture and human health, public concern is increasing towards safety of antibiotic drug usage in aquaculture (e.g., Alderman and Hastings, 1998 and Goldburg *et al.* 2001). The use of antibiotic is still running by many researchers such as Gross and Knowlton (2002) who used 50 ng/ml amphotericin B, as an antifungal agent, plus 50 ng/ml each of the antibiotics streptomycin sulfate and ampicillin to maintain cultures of shrimp *Alpheus heterochaelis* adults. It should be taken into consideration that total abortion of antibiotic use will not occur suddenly. Accordingly, the probiotic alternative solution may be accepted by many farms. Till this time, we planned to focus on evaluation of the antibiotic resistance in some shrimp pathogen and obtained hopeful results. Presence of the probiotic *B. subtilis* could cause good reduction in the resistance of the pathogens to all the used antibiotics. The aminoglycosides streptomycin and gentamicin were highly responsive to addition of *B. subtilis*r. The other four antibiotics (including the commonly used oxitetracycline) were relatively less affected by the probiotic. Finally, it could be said that the presence of probiotics in shrimp cultures is advantageous for competing with the undesirable bacteria in the digestive system, enhancing establishment of the beneficial digestive microflora and hence enhancing growth of shrimp. In addition, it competes the pathogenic bacteria, interferes with the extra- and intracellular proteins of such pathogens and also decreases the antibiotic resistance of the pathogen towards the selected antibiotics.

CONCLUSION

The results obtained from the present study indicated the useful of the utilization of probiotic diets in shrimp larval feeding based on the survival rate, growth rate and its challenge bacteria against to *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio proteolyticus*. It may produce a good quality of shrimp postlarvae to supply shrimp culture systems with biosecure seeds. Therefore, it could be encourage applying this trail in marine shrimp hatcheries.

ACKNOWLEDGMENT

This study was financed by the Regional Council for Agricultural Research and Extension (RCARE), Ministry of Agriculture, Egypt. The authors would like to express their deep thanks to Prof. Mohamad Abed El-Migyd and Prof. Abd Elrahman Hashim for their valuable cooperation.

REFERENCES

Aguirre-Guzman, G., Labreuche, Y. and Ansquer, D. (2003). Proteinaceous exotoxins of shrimp-pathogenic isolates of *Vibrio penaeicida* and *Vibrio nigripulchritudo* Ciencias Marinas, 29(1): 77–88.

Alderman, D.J. and Hastings, T.S.M.(1998). "Antibiotic Use in Aquaculture: Development of Resistance – Potential for Consumer Health Risks." International Journal of Food Science and Technology, 33(2): 139-155.

AOAC (1990). Official Methods of Analysis of Association of Official Analytical Chemists. 15 th Ed .Published by the Association of Analytical Chemists. Virginia 2220/USA.

Baticados, M.C.L, Lavilla-Pitogo, C.R.and Cruz-Lacierda, E.R. (1990). Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis. Aquat. Org., 9: 133-139.

Bryant, T.N. (2002). Probabilistic Identification of Bacteria for Windows. Version 1.9.2. ((http://www.som.soton.ac.uk/staff/tnb/pib.htm)-) - 21/05/02.

Cahu, C.L.; Zambonino, Infante, J.L.; Escaffre, A.M.; Bergot, P. and Kaushik, S. (1998). Preliminary results on sea bass, *Dicentrarchus labrax* larvae rearing with compound diet from first feeding. Comparison with carp (*Cyprinus carpio*) larvae. Aquaculture, 169: 1-7.

Casco, A,N, Yugueros, J.and Temprano, A.(2000). A Major Secreted Elastase Is Essential for Pathogenicity of *Aeromonas hydrophila*. Infect. Immun., 68 (6): 3233–3241.

Davey, M.L., Hancock, R.E.W. and Mutharia L.M. (1998). Influence of Culture Conditions on Expression of the 40-Kilodalton Porin Protein of *Vibrio anguillarum* Appl. Environ. Microbiol., 64 (1): 1138–146.

El-Dakar, A.Y. (2001). Utilization of five marine microalgae species in larval feeding of *Penaeus japonicus*: 1-monoalgae. In Hendry,C.I.; Van Stappen, G.; Wille, M.and Sorgeloos, P. (Eds.), Larvi 2001-Fish and Shellfish Larviculture Symposium, Special Publications, vol. 30. European Aquaculture Society, Gent, Belgium, pp, 170-173.

El-Dakar, A.Y.; Ghoniem S.I. and Nour A.M. (1999). Optimum protein requirements of *Penaeus japonicus* during weaning stage by using microparticulated diets. Egypt. J. Aquat. Biol. & Fish., 3 (1): 1-16.

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

Gatlin III, D.M. (2002). Nutrition and fish health. In: Halver, J.E. & Hardy, R,W. (*Eds.*), *Fish Nutrition*. Academic Press, San Diego, CA, USA, pp. 671-702.

Goldburg, R.J., Elliot, M.S. and Naylor, R.L. (2001). "Marine Aquaculture in the United States: Environmental Impacts and Policy Options." Report prepared for the Pew Oceans Commission. Arlington, Virginia. Pp.1-9.

Green, D. Wakeley H.,and Anthony, P. (1999). Characterization Of Two *Bacillus* Probiotics. Appl. Environ. Microbiol., 65(9): 94288–4291.

Griffith, D.R.W. (1995). Microbiology and the role of probiotics in Ecadorian shrimp hatcheries. In Lavans. P., Jaspers, E. & Roelants, I. (Eds.), Larvi 95-Fish and Shellfish Larviculture Symposium, Special Publications, vol. 24. European Aquaculture Society, Gent, Belgium, pp, 478.

Gross, P.S. and Knowlton, R.E. (2002). Morphological variations among larval-postlarval intermediates produced by eyestalk ablation in the snapping shrimp *Alpheus heterochaelis* Say. Biol. Bull. 202: 43–52.

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

Healey, K. (2004). Use of *Bacillus* cultures as probiotic for aquaculture. Abstract Book of Australian Aquaculture Conference: profiting from sustainability, 2004, September 26-29, 2004, Sydney, NSW Australia, pp 155.

Hoefer Scientific Instruments (1992-1993). SDS Gels, San Francisco, USA.). Influence of polarisation and differentiation on interaction of 43-kDa outer-membrane protein of *Aeromonas caviae* with human enterocyte-like Caco-2 cell line. Interntl. J. Molecular Med., 11: 661-667.

Irianto, A. and Austin, B. (2002). Probiotics in aquaculture (review). Journal of Fish Diseases, 25 (11): 633

Janda, J.M. (1985). Biochemical and exoenzymatic properties of *Aeromonas* species. Diagn. Microbiol. Infect. Dis., 3: 223–232.

Joanne, M.R., Houston, C.W. and Kurosky, A. (1989). Bioactivity and immunological characterization of a cholera toxin-cross-reactive cytolytic enterotoxin from *Aeromonas hydrophila*. Infect. Immun. 57:1170–1179.

Kolkovski, S. (2001). Digestive enzymes in fish larvae and juveniles-implications to formulated diets. Aquaculture, 200: 181-201.

Koven, W.M.; Parra, G.; Kolkovski, S. and Tandler, A. (1998). The effect of dietary phosphotidylcholine and its constituent fatty acids on microdiet ingestion and fatty acid absorption rate in gilthead seabream, *Sparus aurata* larvae. Aquaculture Nutrition, 4: 39-45.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227: 680-685.

Lavens, P. and Sorgeloos, P. (1996). Manual on the production and use of the live food for aquaculture. FAO, Fisheries Technical paper 360, No. 361. Rome.

Li, P. and Galtin III, D.M. (2004). Dietary brewers yeast and the prebiotic Grobiotic™AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* X *M. saxatilis*) to Streptococcus iniae infection. Aquaculture, 231: 445-456.

Lowry, O.H., Rosebough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

MacMillan, J.R. (2001). Aquaculture and antibiotic resistance: A negligible public health risk. World Aquaculture. 68: 49-51.

Millership, S.E. and Want, S.V. (1993). Characterization of Strains of Aeromonas spp. by Phenotype and Whole-Cell Protein Fingerprint. J. Med. Microbiol., 39: 107-113.

Moriarty, D.J. (1998). Control of luminous *Vibrio* specis in penaeid aquaculture ponds. Aquaculture, 164: 351-358.

National Committee for Clinical Laboratory Standards,NCCLS(1990). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7- T2. Villanova.

National Research Council, NRC (1993). Nutrition Requirements of Fish. National Academy Press. Washington, D.C. 114pp.

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

Rengpipat, S Rukpratanporn, S.; Piyatiratitivorakul, S. and Menasaveta, P. (2000). Immunity enhancement in black tiger shrimp *Penaeus monodon* by a probiont bacterium (*Bacillus cereus*. Bacteriological Analysis Manual, 8th ed. U.S. Food and Drug Administration, U.S. Department of Health Human Services, USA. Revision A. Chapter 14.

Rosenberry, R. (1998). World Shrimp Farming, 1998. Annual Report, Shrimp News International.

Snedecor, G.W. and Cochran, W.G. (1982). Statistical Methods, 6 th ed. Iowa State University. Press, Ames. Iowa.

Southgate, P.C. and Partridge, G.H. (1998). Development of artificial diets for marine finfish larvae: problems and prospects In: DeSilva S.S (ed.). Tropical Mariculture. Academic Press, London, pp 151-169.

Sugita, H.; Hirose, Y.; Matsuo, N. and Deguchi, Y. (1998). Production of the antibacterial substance by Bacillus sp strain NM12, an intestinal bacterium of Japanese costal fish, Aquaculture, 165: 371-378.

Takeuchi, T. (2001). A review of feed development for early life stage of marine finfish in Japan. Aquaculture, 200: 203-222.

Tanasomwang, V.; Nakai, T.; Nishimura, Y. and Muroga, K. (1998). Vibrio-inhibiting marine bacteria isolates from black tiger shrimp hatchery. Fish Pathol., 33: 459-466.

Teshema S.I., Kanazawa K. and Sakamoto M. (1982). Microparticulate diets for the larvae of aquatic animals. Mir. Rev. Data File Fish. Res., 2: 67-86.

Whittington, R.J., Gudkovs, N., Carrigan, M.J., Ashburner, L.D. and Thurstan, S.J. (1987). Clinical, Microbiological and Epidemiological Findings in Recent Outbreaks of Goldfish Ulcer Diseases due to Atypical *Aeromonas salmonicida* in South-Eastern Australia. J. Fish Dis., 10: 353-362.

Wilson R.P. (1989). Amino acids and proteins. In: Fish Nutrition. 2 nd edition (Halver J.E., ed.) pp:112-152.

Wyban J. (2000). Breeding shrimp for fast growth and virus resistance. Advocate, 3 (6): 32-33.

Wyban, J.A.; Swingle, J.S.; Sweeney, J.N. and Pruder, G.D. (1992). Development and commercial performance of high health shrimp using specific pathogen free (SPF) broodstock *Penaeus vannamei*. Proceedings of the Special session on Shrimp Farming. The World Aquaculture Society, Baton Rouge, pp. 254-260.

استخدام بكتريا الباسيلس فى علائق الحبيبات الصغيرة للإنتاج الآمن حيويا للجمبرى اليابانى

أشرف يوسف الدكر[١] ، يسرى محمود جوهر[٢]

١- كلية العلوم الزراعية البيئية بالعريش-جامعة قناة السويس

٢- شعبة الميكروبيولوجى-كلية العلوم -جامعة الاسكندرية

أجريت تجربتين غذائيتين منفصلتين لدراسة ناثير استخدام بكتريا الباسيلس ستلس فى العلائق الصغرى على معدل الاعاشة والنمو ومقاومة الامراض للجمبرى اليابانى خلال مرحلتي اليرقات وما بعد اليرقات. أجريت التجربة الاولى على نطاق تجريبى وذلك باستخدام ستة تنكات دائرية ذات قاع مخروطى مصنوعة من الفيبرجلاس (١٠٠ لتر) لتخزين ١٠٠٠ وحدة PL لكل تنك. أما التجربة الثانية فقد أجريت على نطاق تطبيقى أستخدم فيها تنكات فيبرجلاس على شكل حرف U بحجم ٣,٦ م^{٣} وخزن بكل منها ١٨٠ ألف وحدة يرقة جمبري فى مرحلة Nuplius stage . وقد تم تغذية اليرقات بعليقة تحتوى على ٥٣% بروتين مع أو بدون بكتريا الباسيلس والتى أضيفت بمعدل ٥٠٠ مجم/١٠٠جم (١٠X٧^{٧} خلية/جم) وذلك فى مرحلة PL_1 للتجربة الأولى وفى مرحلة M1 فى التجربة الثانية. كما تم دراسة مقدرة البكتريا على مقاومة ثلاثة ميكروبات مرضية هى:

Aeromonas hydrophila, *Edwardsiella tarda*, and *Vibrio proteolyticus*

كان معدل الإعاشة للجمبرى الذى تغذى على العليقة المحتوية على البكتريا مرتفع عن تلك التى تغذت على العليقة الاساسية فى كلا التجربتين. بالإضافة الى أن البكتريا قد حسنت النمو إذا ما قورنت المجموعة الكنترول. وقد لوحظت تغيرات كبيرة ومؤثرة فى تركيب البروتين داخل أو خارج الخلايا للميكروبات المرضية الثلاث عندما حللت كروماتوجراقيا SDS-PAGE electrophoresis قبل أو بعد معاملتها بالبكتريا. وكذلك انخفض بشدة نشاط الإنزيمات المحللة للبروتين فى الميكروبات المرضية بتعرضها للافرازات الخارجية لبكتريا الباسيلس. وأظهرت بكتريا الباسيلس ستلس مقدرة كبيرة فى مقاومة الـ *Vibrio* يليه الـ *Aeromonas* ثم الـ *Edwardsiella*. لذا توصى هذه الدراسة بأهمية استحدام الباسيبس ستلس فى غذاء الجمبرى.