9

Probiotics in Aquaculture of Kuwait – Current State and Prospect

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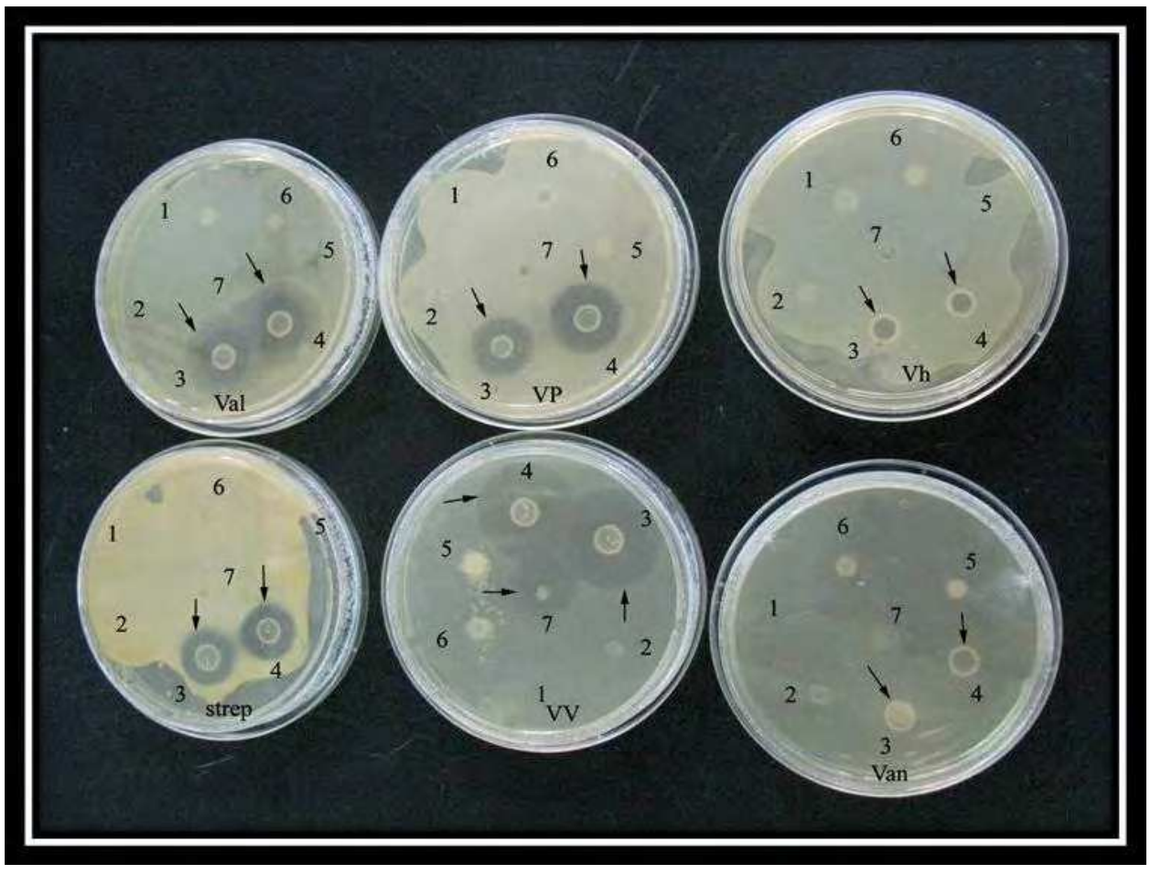
1. Introduction

The blue-fin porgy, *Sparidentex hasta,* known as sobaity in Kuwait, is a commercially valuable food fish greatly preferred in Kuwait and other Arabian Gulf countries. This species has been cultured at the Kuwait Institute for Scientific Research (KISR) since 1979 (Hussain et al., 1981). From 1982 to1986, research efforts were carried out with the intension of developing a commercially valid culture technology for sobaity (Teng et al., 1984). The research efforts were completed, and the results were assessed to formulate a culture technology recommended for commercial application (Teng et al., 1999). However, a number of bacterial diseases have been reported that cause severe losses in sobaity larvae. Outbreaks of vibriosis caused by Gram-negative bacteria *Vibrio* spp., is the most serious bacterial disease of both grouper and sobaity (Rasheed, 1989a). They were identified as *V. anguillarum*, *V. ordalli* and *V. carchariae* and *V. damsela*. In addition, *V. harveyi* was associated with mortalities in hamoor and sobaity (Saeed, 1995). So far, conventional approaches, such as the use of antimicrobial drugs, have had limited success in the prevention or cure of aquatic animal disease. Furthermore, there is a growing concern about the use and, particularly, the abuse of antimicrobial drugs in aquaculture. The practice of using antibiotics indiscriminately for the treatment of diseases in aquaculture could result in the accumulation of residues and the development of resistant strains of bacteria (Uma, 1999). This leads to the search for new, more effective antibiotics thus increasing the consumption of antibiotics in aquaculture. Vaccination can not prevent the development of the disease in young and small fish (Ellis, 1999; Magnadottir et al. 2006). Conventional vaccination is, accordingly, not of value before this time, and the larvae are wholly reliant on the non-specific immune parameters. Thus, an alternative methods are to be evolved to maintain a healthy microbial environment in fish rearing tanks. One such method that is gaining recent acceptance within the aquaculture industry is the use of probiotics bacteria to control potential bacterial pathogens (Wang et al., 2008; Decamp et al., 2010). Thus, the application of probiotics may provide a potential alternative method to protect fish from infectious diseases and improve the survival of cultured marine fish (Irianto and Austin, 2003). The aim of the present study was to evaluate the effect of three autochthonous probiotics, coded as SHBP, 4SQ and 5L8 and a standard isolate *Lactobacillus divergens* (ATCC, 35677) on growth performance of rotifers and the effect of dietary administration of SHBP, *L. divergens* and a combination (SHBP& *L. divergens*) on the survival rate of sobaity larvae.

2. Screening, isolation and *in vitro* antagonism test of autochthonous probiotics

Performing an *in vitro* antagonism test is considered to be an important step in screening potential probiotics, in which pathogenic bacteria are exposed to the selective probiotics in liquid **(**Gildberg, et al., 1995, 1997) or solid (Austin et al., 1992; Dopazo, et al., 1988; Westerdahl et al., 1990) medium. The preselection of candidate probiotics based on these *in vitro* antagonism tests has usually led to the finding of effective probiotics (Verschuere et al., 2000). Bacterial isolates were obtained from the mid and hind gut of cultured yellow-fin porgy *Acanthopagrus latus* (shaem), wild silver pomfret *Pampus argenteus* (zobaidy), wild orange-spotted grouper *Epinephelus coioides* (hamoor), wild tigertooth croaker, *Otolithes argenteus* (newaiby), cultured blue- fin porgy *Sparidentex hasta* (sobaity), *Lactobacillus* sp., (Alken-Clear FIO-1006, Alken-Murray Corp, USA) and a *Lactobacillus divergens* (ATCC, 35677). Autochthonous probiotic, Gram-positive bacteria isolated from cultured shaem was coded as SHPB, form wild zobaidy, coded as 4SQI, from cultured sobaity coded as S24, form wild newaiby coded as 5L82 and from wild hamoor coded as 5M99b. Probiotic bacteria were cultured in brain heart infusion broth (BHIB, Oxoid, Basingstoke, UK) with 2% NaCl. After cultivation, bacteria were harvested by centrifugation (2000 rpm for 10 min), washed twice and resuspended in phosphate-buffered saline (PBS). The *in vitro* antagonism of the isolated autochthonous probiotics against *Vibrio alginolyticus* (locally isolated from diseased cultured sobaity), *V. anguillarum* (ATCC 43310), *V. harveyi* (locally isolated from diseased cultured mullet *Liza klunzingeri*), *V. parahaemolyticus* (ATCC, 27159), *V. vulnificus* (ATCC, 33149) *and Streptococcus agalactiae* (locally isolated from diseased zobaidy) was investigated. The inhibitory activity was assessed by three antagonism tests, the first test is the well-diffusion test (Perez et al., 1990) and the second test is the double-layer method (Dopazo et al., 1988). The third test was a modification to the double-layer method. The new modified technique applied was named as "filter paper disc method", which aimed to obtain precise and accurate inhibition zones created by the probiotics bacteria against the pathogenic bacteria. During the current research the third method was selected. Five sterile filter paper disks were placed on the BHIA, and a drop of the probiotic culture (2 µL) was placed on the sterile filter papers, and incubated overnight at 30ºC. The overnight cultures of the pathogenic bacteria were prepared at 1:10 dilution using sterile phosphate buffered saline. All the probiotic cells were killed by exposing the plate to each diluted pathogenic bacteria were overlayed. The cultures were incubated overnight at 30 ºC and the zone of inhibition was measured. The results showed that all the methods were suitable to assess the effect of probiotic bacteria on pathogenic bacteria. However, the modified overlay method seems to be more effective for the selection of probiotics. This method showed consistent results on the zone of inhibition compared to the other two methods that some times produced doubtful false negative results mainly due to the swarming nature of growth in the case of *V. alginolyticus*. In addition, the administration of suitable concentration of probiotics and allowing growth and production of antimicrobial compounds before the addition of *Vibrio* spp produced a reliable inhibition results. The probiotics coded as SHPB and 4SQI showed a significant zone of inhibition against all the pathogenic bacteria (Plate1). The other probiotics showed some effect, but they were unable to inhibit the growth of all the pathogenic bacteria.



1: Lactobacillus sp; 2: Lactobacillus divergens; 3: 4SQI; 4: SHPB; 5: 5L82; 6: M99b; 7:S24.
Val: V. alginolyticus; VP: V. parahaemolyticus; Vh: V. harveyi; Strep: Streptococcus agalactiae; VV: V. vulnificus; Van: V. anguillarum.

Plate 1. Antagonism test of seven putative probiotics against six pathogenic bacteria by the modified double-layer method.

3. Competitive exclusion of vibrio co-cultured with autochthonous probiotics (SHPB)

Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques & Arevalo, 1995). It has been reported that bacterial strains associated with intestinal and skin mucus of adult marine turbot *Scophthalmus maximus* and dab *Limanda limanda*, suppressed the growth of the fish pathogen *V. anguillarum* (Olsson et al., 1992). Thus, the manipulation of microbial constitutes is a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcazar et al., 2006). In this study, co-culture of *Vibrio* sp. and SHPB was plated on a BHI agar plate. A 24 h BHI broth culture of *Vibrio* spp., and SHPB was used. The suspensions of individual bacteria after harvesting and adjusting the cell density to 10^4 / mL were used. Both the suspensions were spread (100 µL each) on a BHI agar plate and incubated for observing the colony formation. The colonies were observed, under the microscope, at 0, 3 and 6 h of incubation at 30°C for competitive exclusion or invasive growth. The SHPB showed a distinctive competitive exclusion against *Vibrio* spp., (plate 2).

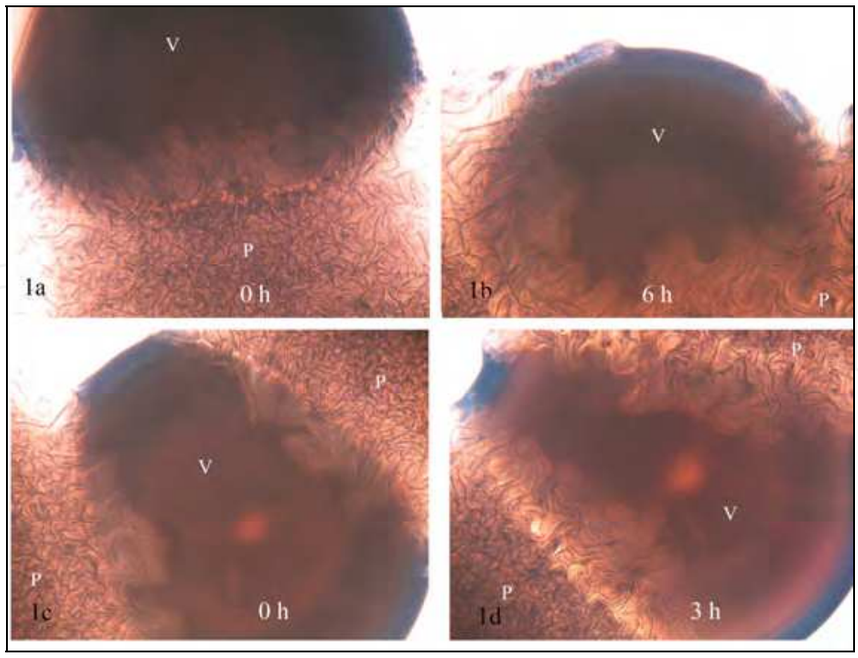


Plate 2. Competitive exclusion of SHPB (P) against one pathogenic Vibrio (V) After 6 h (1a &1b) and after 3 h (1c & 1d). 20 x.

4. Effect of autochthonous probiotics on rotifer proliferation

Several studies related to marine fish larviculture have attempted to find suitable probiotics that has a positive effect on the live food and improves their dietary value (Benetti et al., 2004; Robertson et al., 2000; Gomez-Gil et al., 2000; Ringo and Birkbeck, 1999; Harzevilli et al., 1998; Gatesoupe, 1991a). Douillet (2000) reported that rotifers cultured with an *Altermonas* strain or blend of strains resulted in a consistent enhancement of the rotifers culture. In this study, rotifers *Brachionus plicatilis* were enriched for 24 h with a high quality mixture of algae (Nanochloropsis, Tetraselmis and Isochrysis) along with the available commercial enrichments (Super-Selco and DHA protein selco). The rotifer density was 22 cell/ mL. A one week experiment was carried out to evaluate the proliferation of the rotifers treated with probiotics SHPB, 4SQI, 5L82 and *L. divergens*. Daily 1 mL of the probiotic and 1 mL of the algal suspension was added to each flask, which corresponds to 10^{10} CFU/ mL. The control flasks received 2 mL algae daily. The rotifer count was determined for seven days to assess the effect of the probiotics. An increase in rotifers proliferation and reproducibility within different treatments was obtained, as described by the differences in the coefficient of determination for four probiotics treatments and the control (Fig. 1). The probiotic SHPB and *L.divergens* showed better enhancement on the cell population with time, while 4SQI and 5L8 showed lower cell proliferation response compared to the control. The SHPB gave the best compared to the control and other probiotics, mainly during the first three days. All the probiotics tested showed better effect on the rotifer counts compared to the control. The *Vibrio* load in the rotifer population without probiotic treatment was significantly higher than that of the probiotic treated sets during the first three days. However, the vibrio population was significantly lower in SHPB treated rotifer all through the week. The sobaity larvae usually start feeding on day two or three post hatch. Thus, the

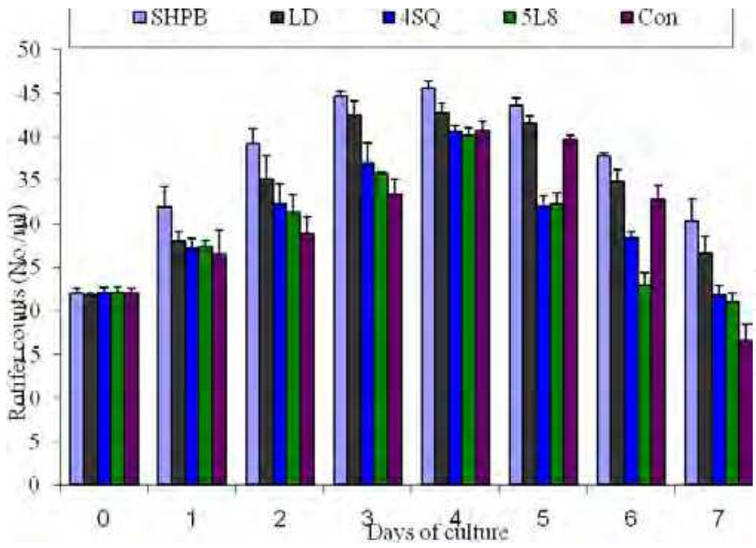


Fig. 1. The effect of SHPB (*B. halotolerance*), LD (*L. divergens*), 4SQI, 5L82 on the growth performance of rotifers compared with the control (Con).

rotifers are initially added on these days with a starting density of 5 rotifer/ mL. The probiotics were added daily, this could explain the significant effect mainly for the SHPB on rotifers proliferation all through out the experiment. Repetitive addition of the probiotics can significantly enhance the rotifers performance and survival. The result of this study is in agreement with the results obtained by Planas et al., (2006). They reported that continuous additions of probiotic (*Rosebacter* strain) are necessary to maintain a minimum level of it in the rotifer and the culture water. In addition, exposing the larvae to sufficient probiotic concentrations will increase the chance of the probiotics being ingested by the larvae. Thus, this could lead to the advantage of improving the survival rate of the fish larvae.

5. Effect of SHPB and *L. divergens* used alone and in combination (SHPB & *L. divergens*) on the survival of sobaity larvae

Owing to the problem of antibiotic resistance and subsequent reluctance of using antibiotics, the use of probiotics in larviculture is becoming increasingly popular. During the early stages of development, manipulation of the larval digestive system seems possible through the addition of probiotics, either through the culture water, or via the live food (Vine et al., 2006). Geovanny et al. (2007) reported that the use of probiotics can increase the survival rate and control the high incidences of larval diseases. Thus, by shifting the bacterial flora in live feed organisms to probiotic species, this can assist the fish larvae to minimize the pathogenic bacteria from the feed, and the fry will benefit from the probiotic bacteria. Several bacterial probiotics were used in the larval culture of aquatic organisms. Kozasa (1986) reported that the spores of *Bacillus toyoi* increased the growth rate of yellowtail and reduced the mortality of Japanese eel that were infected by *Edwardsiella* sp. The Gram-negative *Vibrio pelagius* decreased the mortality of the turbot larvae *Scophthalmus maximus* challenged with *Aeromonas caviae* (Ringo and Vadstein, 1998). Gatesoupe (1991b) showed that *Bacillus toyoi* and *Bacillus* sp spores increased the growth rate of larval turbot introduced via the rotifer *Brachionus plicatilis*. Pirarat et al., (2006), reported that supplementation of

L. rhamnosus significantly reduced cumulative mortality due to *E. tarda*, confirming the protective effect of a probiotic bacterium against this pathogenic bacterium. Suzer et al., 2008 showed significant increase in the survival rate of *Sparus auratus* larvae fed with *Lactobacillus* spp. via live food and water. In this study, the effect of single and combined administration of SHPB and *L. divergens* on the survival rate of sobaity larvae was evaluated. The larvae were reared in 1m^3 circular fiberglass tanks with stocking density of 60 larvae/ L. The enriched rotifers were added to the larval tanks and the rotifer density was maintained at 5/ mL. The survival rate of the larvae was determined at 28 days post hatch. The results showed that all the treatment with probiotics significantly enhanced the survival rate compared to the control. The survival in *L. divergens* fed larvae was the highest (11.7%) whereas it was 9.2% in the mixed probiotics, 8.9% in SHPB and 6.3% in the control (Fig 2). Balcazar et al., 2007, demonstrated that the administration of a mixture of bacterial strains (*Bacillus* sp. and *Vibrio* sp.) positively influenced the growth and survival of white shrimp juvenile. Salinas et al., 2008, showed that the combined probiotics, *L. delbruekii* and *B. subtilis* enhanced the cellular innate immune system of gilthead seabream. Suzer et al., 2008, showed that *Sparus aurata* larvae fed with commercial *Lactobacillus* spp via live food increases survival rate and specific growth rate. To our knowledge there have been no studies on any probiotic on the blue- finned *Sparidentex hasta* (sobaity). So, in our study, since all the tested probiotics showed significant survival rate compared to the control, the dietary administration of both singly SHPB and *L.divergens* and in combination (SHPB and *L. divergens*) were used and they showed significant increased in the survival rate of sobaity larvae and seems to be a promising probiotic candidate. However, feeding sobaity larvae with a combination of them needs further dose adjustment to achieve the best survival rate and possible beneficial interaction between both bacteria in sobaity gut microenvironment, which may make the use of a mixture of different bacterial strains more interesting than using a single bacterium.

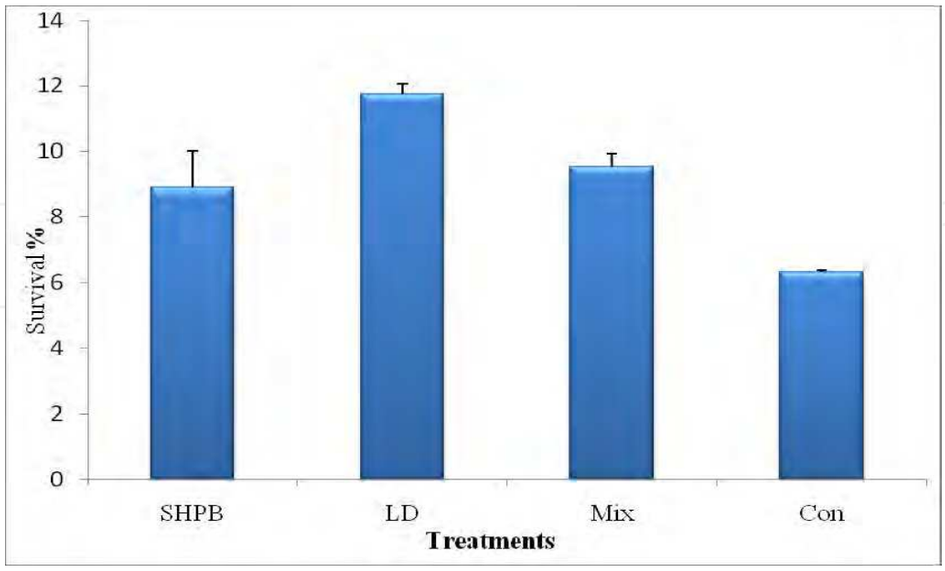


Fig. 2. The survival rate of sobaity larvae fed with SHPB (*B. halotolerance*), LD (*L. divergens*) and their combination (Mix) compared with a control (Con) group fed with un-supplemented diet.

6. Pathogen challenge test for probiotic-fed sobaity larvae

Increased resistance to the pathogen by probiotics has been extensively reported. Growth inhibition against pathogens by *Carnobacterium* was reported (Joborn et al., 1997). The tolerance of rainbow trout *Oncorhynchus mykiss* to furunculosis was enhanced when fed with a diet including the probiotic *L. rhamnosus* (Nikoskelainen et al., 2001). In Atlantic cod *Gadus morhua*, tolerance to *V. anguillarum* increased by feeding with lactic acid bacteria *Carnobacterium divergens* supplemented in the diet (Gildberg et al., 1998). Robertson *et al* ., 2000) reported that Atlantic salmon *Salmo salar* and rainbow trout *O. mykiss*, fed with *Carnobacterium* spp. supplemented in the diet were more tolerant to disease. Chiu et al., 2010, showed that *S. cerevisiae* colonized the intestines of the grouper *E. coioides* fed *S. cerevisiae*-supplemented diets improved and increased the resistance to challenge by *Streptococcus* sp. and a grouper iridovirus. In our study, after feeding the larvae with probiotic-enriched rotifers (SHBP/ *L. divergens* / mixture of SHBP and *L. divergens*) for 28 days, the larvae challenged through immersion against virulent *Vibrio harveyi*. The bath suspension contained a cell density of 10^7 cells m^{-1} and the duration of challenge was 30 min. Fish larvae were transferred to 50 L aquarium tanks (50 fish/ tank) after the challenge, mortalities were observed and recorded for one week and the squash preparations of washed freshly dead larvae were plated on TCBS agar to record specific mortalities. The results showed that fish larvae fed with SHPB and combination probiotics showed clear disease resistance as indicated by distinctive 85.3% and 58% survival rate for SHPB and mixed treatments respectively compared to *L.divergens*-fed fish 54% and control 50%. Pieters et al., 2008, reported that challenge with *A. bestiarum*, the probiotics GC2 and BA211 led to 76% and 88% survival, respectively, in contrast to 22% survival for the controls. In the current study, it is apparent that SHPB and the mixed probiotics (SHPB & *L. divergens*) fulfilled the major requirements of being an effective probiotics by enhancing the survival rate of sobaity larvae after challenge with virulent *V. harveyi*. However, the survival of larvae fed a combination of probiotics was not as good as that of SHPB and here, probably the dose structuring needs to be optimized for furthering the effects of a combination of probiotics (Fig 3).

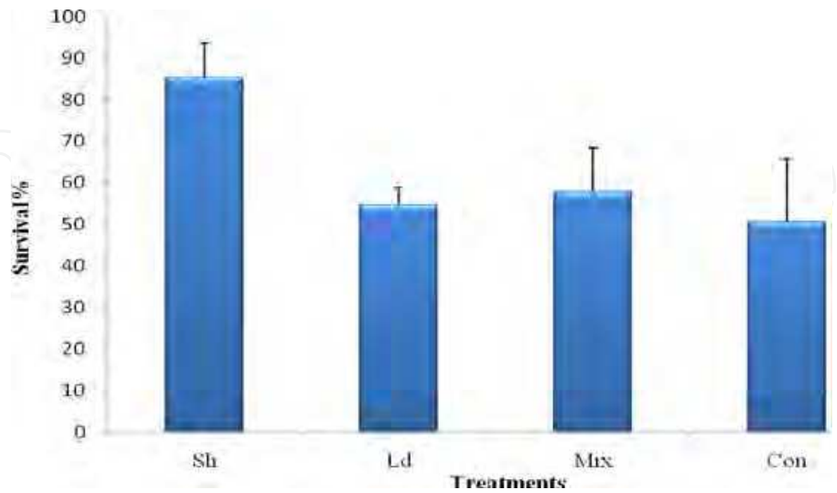


Fig. 3. The survival rate of sobaity larvae challenged against *V. harveyi* after feeding with SHPB (*B. halotolerance*), LD (*L. divergens*) and their combination (Mix) compared with a control (Con) group fed with un-supplemented diet.

7. Molecular characterization of SHPB probiotic and bacteriocin-like compound

The SHPB was characterized using the PCR and 16s rDNA gene amplification (Al-Marzouk et al., 2009). The identification of SHBP probiotic confirmed as *Bacillus halotolerance*. The modes of action of probiotics include the inhibition of a pathogen through the production of bacteriocin-like compounds, competition for attachment sites, competition for nutrients (particularly iron in marine microbes), alteration of enzymatic activity of pathogens, immunostimulatory functions, and nutritional benefits such as improving feed digestibility and feed utilization (Kesarcodi-Watson et al., 2008; Fuller, 1989). Thus, an understanding of the mechanisms probiotics use to compete with pathogens is important when designing a protocol for their selection. Bacteriocins are antibacterial proteins produced by bacteria to kill or inhibit the growth of other bacteria (Cleveland et al., 2001). They are ribosomally synthesized unlike antibiotics, which are synthesized by other mechanisms (Brock & Madigan, 1997). In this study, *B. halotolerance* (SHBP) cultures of different age (12h, 24h, 36h and 48h) were used for detecting the possible role of bacteriocin in the antibacterial activity. The mode of action of *B. halotolerance* was confirmed through its ability to produce bacteriocin-like compound, which is considered as a significant criterion of the defense system displayed by it. It produced an amplicon of approximately 1500 bp and for the bacteriocin gene a 1000 bp amplicon (Plate 3). Cultures of different age, however, showed interesting amplification pattern with clear amplification of the bacteriocin-like gene in 24-h culture and a very mild amplification in 12-h culture. This state was tested by treating the compound (probiotic bacteria free BHI broth) with different pH and temperatures. Later, the treated broth was used in antibacterial assay. The persistence of antibacterial activity of the

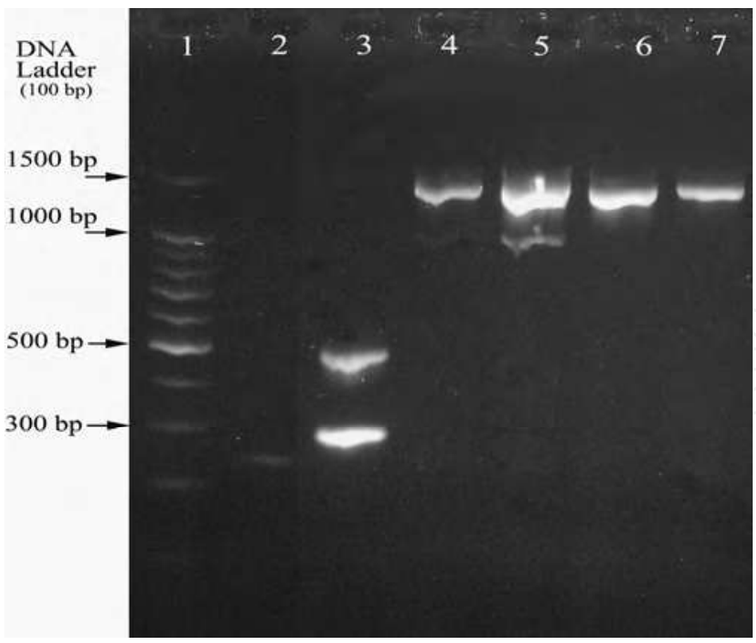


Plate 3. Polymerase Chain Reaction (PCR) amplification of the bacteria specific 16s rDNA (approximately 1500 bp, brighter band) and bacteriocin gene (approximately 1000 bp).
Lanes: 1: 100 bp DNA ladder; 2: Negative sample; 3: Positive amplicon (kit); 4 to 7– probiotic bacterial cultures (12h, 24h, 36 h and 48h) of *B. halotolerance*.

treated broth confirmed that it a bacteriocin- like compound of the probiotic that was responsible for the antagonism. One of the most well known bacteriocins is nisin, which is a ribosomally synthesized antimicrobial peptide produced by certain strains of *Lactococcus lactis* which has been proved to act against human multidrug resistant pathogens such *Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and others (Gatesoupe, 2008). Further research will be required to specify the exact type of bacteriocin produced by the probiotic *B. halotolerance*.

8. Persistence of probiotics in the fish gut

Adhesion and colonization are important for selection and use of probiotic strains (Bussarin & Rakshit, 2006; Vine et al., 2004; Olsson et al., 1992) and because it is considered a pre-requisite for colonization (Beachey, 1981). Adhesion of probiotic bacteria to the intestinal mucosa has been shown to enhance their antagonistic activity against pathogens (Coconnier et al., 2003). In this study, the ability of SHPB, *L. divergens* and their combination to attach to fish intestinal mucus were examined. Colonization of probiotics fed through diets was monitored at 15 and 30 d during the probiotic feeding and at one month after the probiotic-enriched feed was withdrawn. Colonization of the SHPB was evident even at 15 d (Plate 4). The intense localization on the brush boarders of the hindgut intestinal epithelium noticed at 15 and 30 d (Plates 4 & 5) during feeding, suggests that the SHPB is more likely to

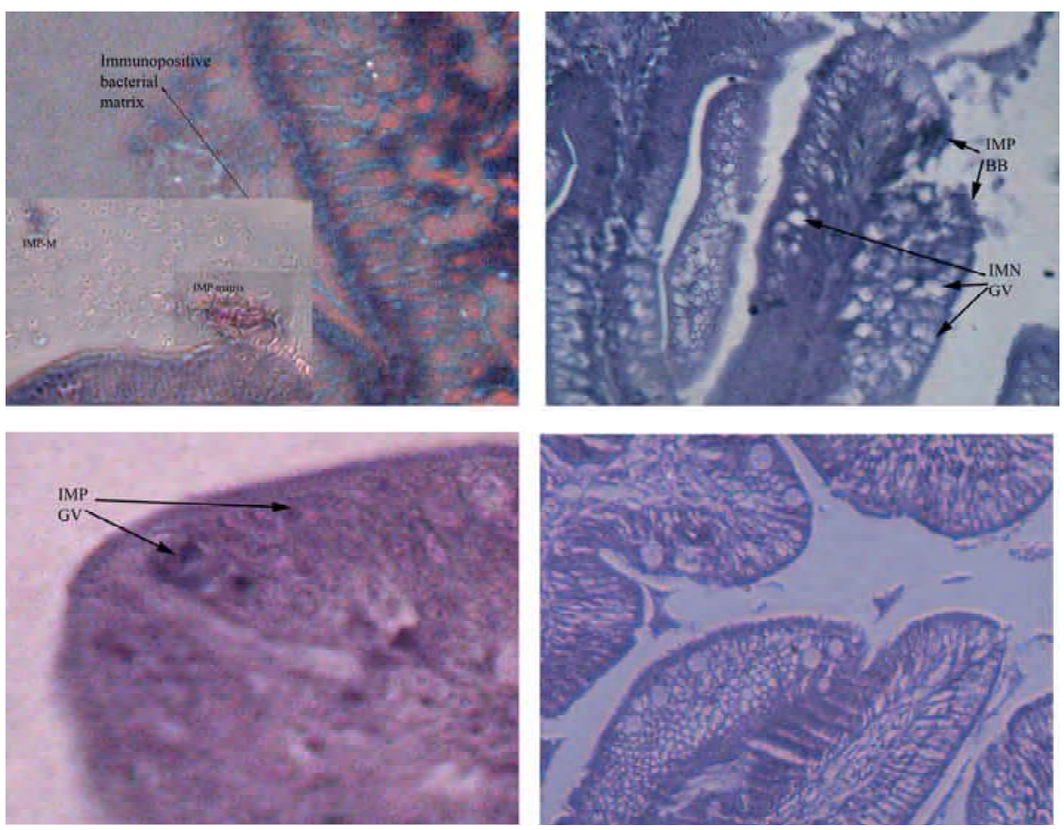
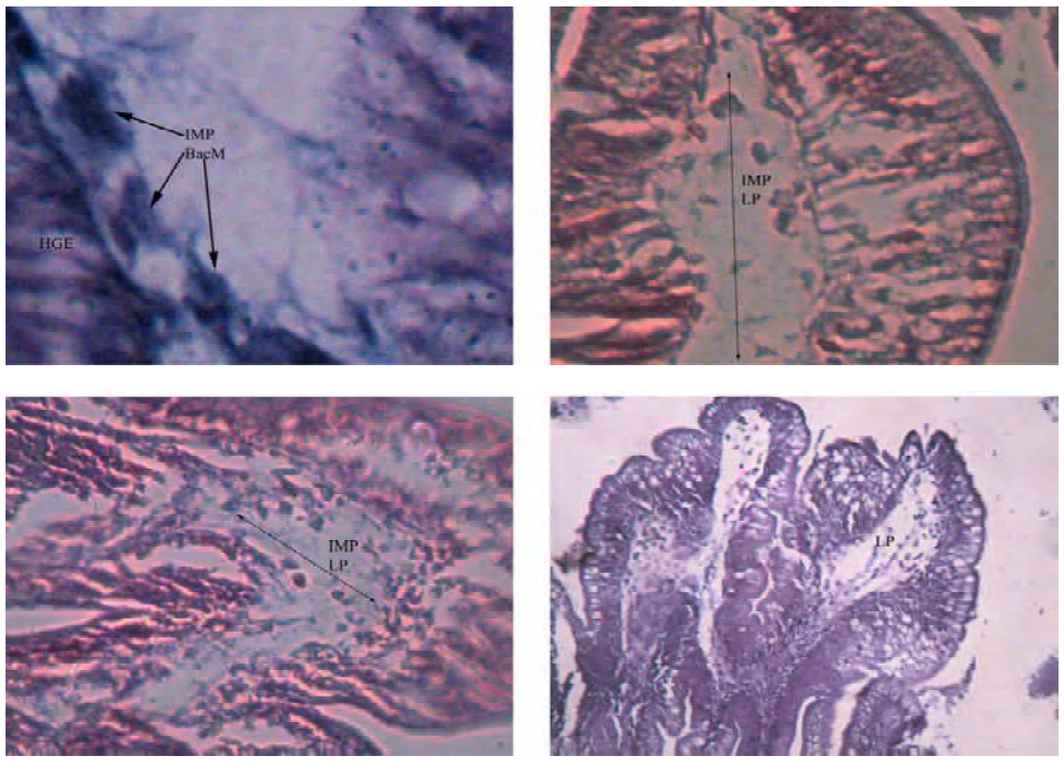


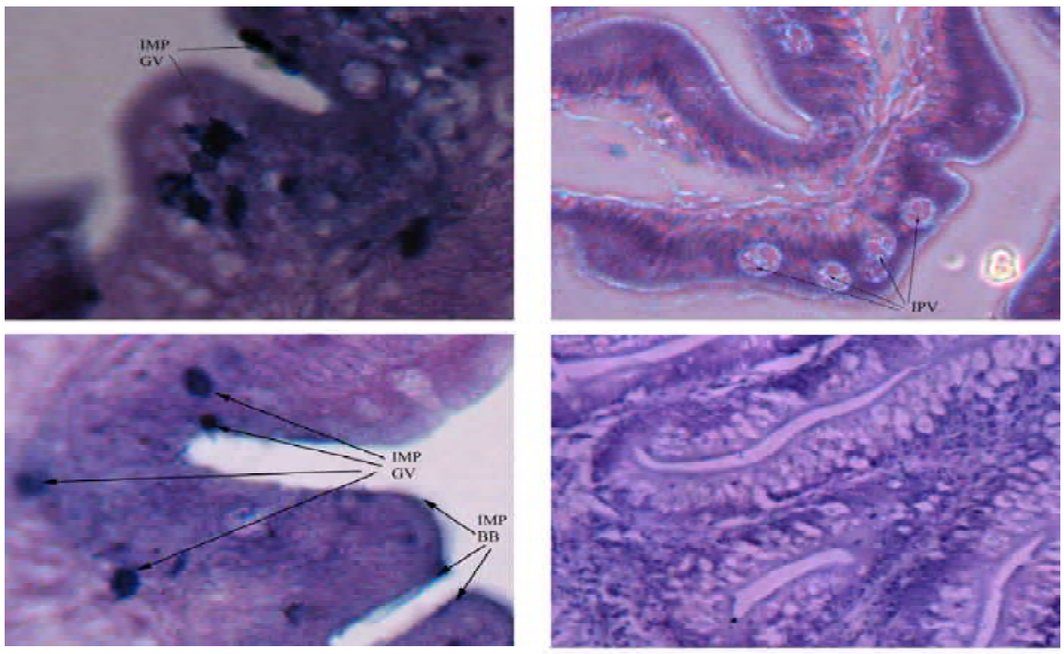
Plate 4. Sobaity gut fed with SHPB, L. divergens & mixed (SHPB & L. divergens) and control diets. Arrows: IMP: Immunopositive; GV: Gut vacuole; BB: brush boarder. Left (40 x) and right. 10 x.



Arrows: HE: hindgut epithelium; LP= lamina propria of the hindgut.

Plate 5. Gut of sobaity sampled after 30 days of continuous feeding with SHPB and L. divergens-(top right, 20 x), mixed probiotics (bottom left, 40x) and control (bottom right, 10x) diets. Note immunopositive SHPB colonization on the hindgut epithelium (top left). No immunopositive bluish-purple reaction in the gut of control fish (bottom right, 10x).

colonize in the hind gut of sobaity than the *L. divergens*, which was processed as an antigen and did not remain in the gut long enough (almost no localization at one month post withdrawal). Clear and significant immunopositive localization (Plate 4) was noticed in gut of both probiotic SHPB and mixed (SHPB and *L. divergens*) probiotic-fed sobaity even after withdrawal of probiotic feeding. This indicates the higher persistence of the SHPB than that of *L. divergens*. The process of colonization is characterized by attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells. Adhesion and colonization of the mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients, or immune modulation (Balcazar et al., 2006). However it needs to be accepted that the efficiency of a selected probiotic *in vitro* may significantly change when administered to the host because it is influenced by more complex factors such as the selective ingestion and the death in the intestinal tract (Vine et al., 2006) caused by the failure of the probiotic to maintain its *in vitro* physiology under circumstances of a more complex microbial interactions and/or nutritional environment. In general, there is a sense of the lack of correlation between in vitro and in vivo experiments in the latest reviews on probiotic use in aquaculture (Balcazar et al., 2006; Vine et al., 2006). The main claimed mechanisms are: competitive exclusion, digestion enhancement, immune response enhancement, water quality improvement and antiviral effects.



No immunopositive bluish-purple reaction in the gut of control fish (bottom right).
Arrows: IPV: immunopositive vacuoles.

Plate 6. After 30 days of withdrawal of feeding with SHPB-(top left, 40x), L.divergens-(top right, 40x), mixed probiotics (bottom left 40x)) and control (bottom right (40 x)) diets.

9. Bacterial count in gastrointestinal tract in probiotics-fed sobaity

No *Vibrio* sp., was detected in the vibrio selective medium (TCBS), from the fish gut fed from SHPB and mixed probiotics (SHPB and *L. divergens*). The highest bacterial count was counted in the control and *L.divergens* treatment (Table 1). The main bacterial colonies that were detected in the brain heart infusion agar (BHIA) from fish fed with SHBP.

Treatment	Media	45d	60d	75d	105d
SHPB	BHIA	6.30×10^4	1.00×10^3	0.30×10^2	0.80×10^3
	TCBS	-	-	-	-
LD	BHIA	3.20×10^3	4.00×10^2	1.70×10^3	0.90×10^4
	TCBS	-	-	1.00×10^2	15.00×0^4
Mix	BHIA	3.00×10^3	6.60×10^3	6.20×10^3	0.30×10^4
	TCBS	-	-	-	-
Con	BHIA	1.30×10^4	6.50×10^2	4.10×10^3	2.10×10^3
	TCBS	-	-	0.30×10^3	1.00×10^3

Table 1. Bacterial Count from the Gut of Sobaity Fed with SHPB (*B. halotolerance*), LD (*L. divergens*) and their Combination (Mix) Compared with Control (Con).

10. Pathogen challenge test for probiotic-fed sobaity fry

Vendrell et al., 2008 showed that Probiotic supplementation of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* reduced fish mortality significantly from 78% in the control group to 46-54% in the probiotic groups after challenged *with Lactococcus garvieae*. In this study, sobaity fry fed with SHPB and mixed probiotics showed clear disease resistance as indicated by distinctive 100% survival rate compared to *L.divergens* fed fish and control (Table 2). Based on the ability of the SHPB and mixed probiotics to attach to fish gut, the growth of the pathogen in the digestive tract might be suppressed by the candidate probiotics presence.

Treatments	**Survival Rate (%)**
SHBP	100
LD	16.6
Mix	100
Con	66

Table 2. Survival Rate of Control (Con) and Probiotic-Fed (SHPB, LD and Mix) Sobaity Fry after Challenge with *V. anguillarum*

11. Immunological assays

The non-specific immune system can be stimulated by probiotics. Taoka et al., 2006 indicated that probiotics supplied in the rearing water and the diet of fish enhanced the stress tolerance and the non-specific immune system of Japanese flounder, providing them a higher resistance against stress conditions and pathogens. It has been demonstrated that oral administration of *Clostridium butyricum* bacteria to rainbow trout enhanced the resistance of fish to vibriosis, by increasing the phagocytic activity of leucocytes (Sakai et al., 1995). Balcazar (2003) demonstrated that the administration of a mixture of bacterial strains (*Bacillus* sp. & *Vibrio* sp.) positively influenced the growth and survival of juveniles of white shrimp and presented a protective effect against the pathogens *Vibrio harveyi* and white spot syndrome virus. This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity. In this study, custom-production and characterization of rabbit polyvalent antibodies against *B. halotolerance* and *L. divergens* were carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, and in addition, to evaluate both the humoral and cellular innate immunity responses of sobaity fed for 75 d with the above mentioned probiotics. These included the phagocytic activity; lysozyme activity, serum immunoglobulin and alternative complement (ACH50). Different immunological parameters, mainly serum and mucus lysozyme, phagocyte activity and complement were enhanced in sobaity fry fed with *B. halotolerance* and the mixed probiotics for 75 and 105 days.

11.1 Lysozyme activity

Lysozyme has an important role in non-specific immune defense system and it contained in the mucus on the fish body surface, and in plasma and liver. Lysozyme has an antibiotic ability and is released by leukocytes. It can damage bacterial cell walls, especially of Gram-positive and some Gram-negative bacteria (Grinde, 1989). Lysozyme activity varies between species of fish, genetic strains and different pathogens. Several reports showed the effect of

probiotics on lysozyme activity. A significant increase in lysozyme activity was observed in Nile tilapia, *O. niloticus*, fed diets containing *S. cerevisiae* for 21 days (El-Boshy et al., 2010). Gatesoupe (2008) reported that feeding with Gram-positive and Gram negative potential probiotics caused increase in the cellular parameters such as macrophages and enhanced lysozyme activity. Kim & Austin (2006) recorded high gut mucosal lysozyme activity in fish fed with *Caronbacterium divergens* B 33 and *Carnobacterium maltaromaticum* B26. Taoka et al., 2006, also reported an enhanced lysozyme level in tilapia fed with live and dead probiotics and recorded a high survival rate when challenged with *Edwardsiella tarda.* Panigrahi et al., 2004, showed significantly higher lysozyme activity in rainbow trout fed with *Lactobacilllus rhamnosus*. Apart from serum lysozyme content, probiotics can also enhance the lysozyme level in skin mucosa of fish (Song et al., 2006). Lysozyme in fish can be measured either by the turbidimetric method or the agarose plate assay. Each method was developed based on the amount of lysis of the gram positive bacteria *Micrococcus lysodeikticus*. In this study, lysozyme activity in serum was determined according to the method of Demers and Bayne (1997) based on the lysis of the lysozyme- sensitive Gram positive bacterium, *Micrococcus lysodeikticus* (Sigma, St. Louis). With the help of a computer application software, Delta Soft 3 (Biometalics Inc., New Jersey, USA) the equivalent unit of activity of the sample as compared to the standard were determined and expressed as μg ml^{-1} serum. The turbidimetric assay results can be reported by two methods; relative lysozyme activity measured in Units/min, and by comparison to a standard curve generated using purified. Our turbidimetric assay results were reported by relative lysozyme activity which measured in Units/min, and by comparison to a standard curve generated using purified hen egg white lysozyme. The results showed that sobaity fry fed with three types of probiotics has significant higher lysozyme activity (Table 3).

Treatments	ACH50 (log_2) titers		Lysozyme (units/min)			
			Serum		Mucus	
	75d	105d	75d	105d	75d	105d
SHPB	6.67 ± 0.58	5.67 ± 0.58	18.63 ± 4.50	17.00 ± 6.61	21.25 ± 4.53	26.38 ± 7.37
LD	3.67 ± 0.58	4.67 ± 0.58	15.50 ± 4.34	15.38 ± 5.66	18.63 ± 5.15	20.63 ± 4.60
Mix	4.67 ± 0.58	6.67 ± 0.58	20.13 ± 4.26	17.13 ± 7.24	22.38 ± 4.69	20.38 ± 2.26
Con	2.33 ± 0.58	3.67 ± 0.58	11.63 ± 3.81	11.75 ± 2.38	14.13 ± 3.98	12.38 ± 3.50

ACH50 Tukey's HSD=1.35 for treatments; for time HSD= 1.0. ; serum lysozyme HSD= 5.74 for treatments; for time HSD= 4.344; mucus lysozyme HSD= 4.56 for treatments; for time HSD= 3.45. Values less than HSD values considered to be not significant

Table 3. Alternative Complement (ACH50) Titers (log_2), Serum and Mucus Lysozyme Content of Probiotic-Fed Sobaity (75 d) = After Probiotic feeding & (105 d) = After 30 d of Withdrawal of Probiotic feed

11.2 Alternative complement activity (ACH50)

The alternative pathway of **complement activity** has emerged as a powerful nonspecific defense mechanism for protecting fish against a wide range of potentially invasive organisms, such as bacteria, **fungi**, **viruses** and **parasites (Muller-Eberhard, 1988).** Chiu et al., 2010, showed that *Epinephelus coioides* fed *S. cerevisiae*-supplemented diets exhibited

significant increases in both **serum lysozyme** and **alternative complement pathway** activities (ACH50). The same result was also observed in hybrid **tilapia** fed diets containing DVAQUA® diet **(Zohu** et al., 2009). Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, lysozyme, phagocytic activity, alternative complement activity (ACH50) and disease resistance of the grouper *E. coioides* (Son et al., 2009).

Pirarat et al., 2006 reported that *Lactobacillus rhamnosus* probiotic enhanced the ACH50 of tilapia *Orechromis niloticus* and protect the fish from acute septicemic death by *E. tarda*. Balcazar et al., 2007, showed that in comparison to untreated control fish, the alternative complement activity and phagocytic activity of head kidney leukocytes in serum were significantly greater in all probiotic groups (*Lactococcus lactis* ssp. lactis CLFP 100, *Leuconostoc mesenteroides* CLFP 196, and *Lactobacillus sakei* CLFP 202) at the end of the second week of feeding. Our results showed that ACH50 activity was significantly higher in the probiotic-supplemented groups than in the control group after 75 d feeding and 105 d post feeding. After the feeding, the ACH50 activity gradually decreased in the control group, whereas it remained high in both SHPB and the mixed probiotic groups throughout the test period (Table 3). These results indicate that probiotics incorporated in the feed of sobaity resulted in increased serum complement activity and hence enhanced the immunity of the fish against any virulent pathogens.

11.3 Phagocytic assay

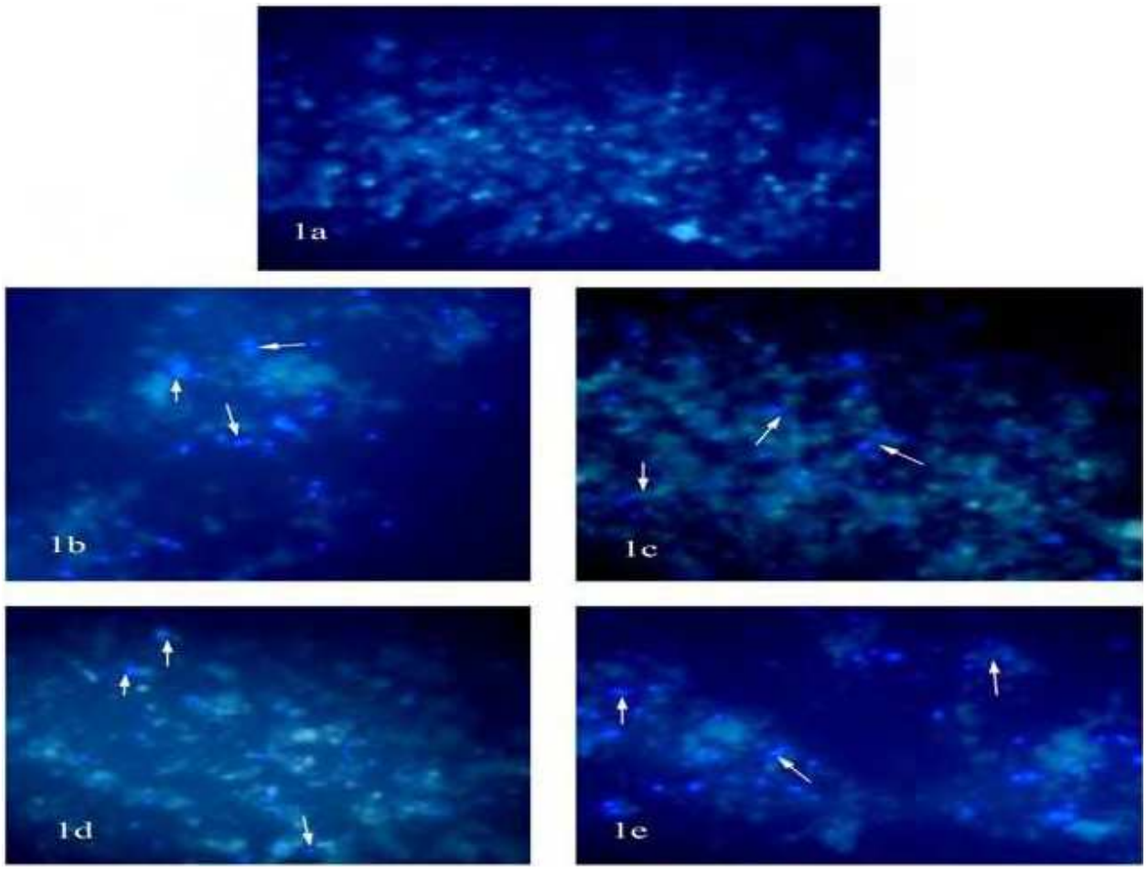
Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses (Nayak, 2010).

Several studies showed the effect of different probiotics on phagocytic activity in several fish species. Probiotics such as *Lactobacillus rhamnosus*, *L. lactis* and *L. acidophilus* can effectively trigger the pahgocytic cells in fish species. These probiotics used either in viable or inactivated form were found to have the ability to stimulate phagocytic activity in different fish species (Brunt et al., 2007; Brunt &Austin, 2005; Irianto & Austin, 2003). Black tiger shrimp fed with a probiotic diet had greater tolerance to *V. harveyi*, and phagocytic activity in hemolymph was activated (Rengpipat et al., 2000; 1998). Sun et al., (2010) reported that phagocytic activity and phagocytic index of grouper fed probiotic *Bacillus pumilus* and *Bacillus clausii* were significantly higher than those of fish fed the control diet for 60 days. Pieters et al., 2008, showed that the analysis of innate immune responses revealed that probiotic GC2, *Aeromonas sobria* promoted higher phagocytic activity in rainbow trout against *Aeromonas bestiarum* after the probiotic was administered orally (10^8 cells per g feed for 14 d). Patricia et al., 2006, reported that the phagocytic ability of gilthead seabream, *Sparus aurata* fed with a mixture of two inactivated bacteria from the Vibrionaceae family was significantly higher than in the control after three weeks feeding. Pirarat et al., 2006, reported that *Lactobacillus rhamnosus* probiotic increases the phagocytic activity of tilapia *Orechromis niloticus* and protect the fish from acute septicemic death by *Edwardsiella tarda*. In this study, the phagocytic ability of head kidney leucocytes was significantly increased after 75 d feeding with SHPB and *L.divergens* supplemented diet. No statistically significant difference was detected in the phagocytic ability of fish fed with a mixture of SHPB and *L.divergens*. The activity fell in a time-dependent manner after 105 d for all the treatments (Table 4). After the completion of 75 d,

Treatments	Phagocytic Index (PI) Number of Fluorescent Beads per Field of Observation	
	FP-PI	EP-PI
SHPB	77.00 ± 13.23	56.00 ± 7.94
LD	65.67 ± 15.67	43.83 ± 5.53
Mix	58.83 ± 20.97	37.17 ± 9.72
Con	43.33 ± 5.13	29.00 ± 2.00

FP= End of probiotic feeding period (75 d) and EP = End of experiment period (105 d).
Treatments Tukey's HSD=22.28; Time Tukey's HSD=16.50. Values less than HSD values considered to be not significant

Table 4. Phagocytic Index of Fresh Head-Kidney Tissue Imprints from Probiotic-Fed (SHPB, LD and Mix) and Control (Con) Sobaity.



1a – Negative control,
1b – head-kidney imprint from control fish,
1c - head-kidney imprint from SHPB-fed fish, 1d - head-kidney imprint from *L.divergens*-fed fish and 1e - head-kidney imprint from fish fed with a 1:1 mix of SHPB and L.d.

Plate 7. Phagocytic assay for sobaity sampled soon after the completion of 75 d of probiotic feeding showing fresh head-kidney tissue imprints after incubation with fluorescent latex beads. Photographed at 20x magnification.

the fresh head-kidney tissue imprints after incubation with fluorescent latex beads showed clear phagocytic ability in both SHPB and *L.divergens* supplemented diet compared to the mixed treatment and control (Plate 3). Thus, phagocytic activity played an important role in sobaity innate immunity as indicated by the phagocytic assay from fresh head kidney tissue imprints and phagocytic index. Our finding indicate that phagocytosis against bacterium can be enhanced by incorporating autochthonous probiotic alone or mixing it with commercial probiont such as *L. divergens*.

12. Conclusion

A protocol for the isolation and selection of candidate probiotic bacteria based on several selective criteria was accomplished. These criteria include *in vitro* antagonism ability of probiotics against pathogenic bacteria, adhesion ability in the intestinal epithelium to compete for attachment sites on the gut wall, improvement of fish survival, disease resistance and immune responses. Results point out a significant effect of *Bacillus halotolerance* (SHBP) on rotifers proliferation compared to other autochthonous (4SQIb, 5L82) and a commercial (*L. divergens*) probiotics. Results showed that *B. halotolerance* **and** *L. divergens* **had a significant positive effect on the survival mainly during day 12 and 22, compared to the control. Dietary administration of combined** *B. halotolerance* **and** *L. divergens* **and single SHPB and** *L. divergens* **showed significant survival rate compared to the control.** Also, the survival rate of larvae fed with these probiotics was improved after challenging with *V. harveyi*, indicating the positive effect of the probiotics used. Molecular characterization confirmed the identification of SHBP as *Bacillus halotolerance*. It also confirmed the ability of *B. halotolerance* to produce bacteriocin-like compound, which is considered as a significant criterion of the defense systems displayed by it.

The probiotics persistence in the gut indicated that they are able to establish themselves in the digestive tract. Also, different immunological parameters, mainly serum and mucus lysozyme, phagocytic activity and complement activity were enhanced in sobaity fry fed with *B. halotolerance* and the mixed probiotics for 75 and 105 d.

These finding can be applied to other potential cultured fish in Kuwait through future research. In addition the most potential autochthonous probiotics will be evaluated in large scale fish production.

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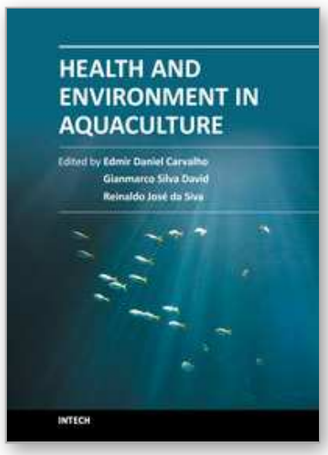

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Aquaculture has been expanding in a fast rate, and further development should rely on the assimilation of scientific knowledge of diverse areas such as molecular and cellular biology, and ecology. Understanding the relation between farmed species and their pathogens and parasites, and this relation to environment is a great challenge. Scientific community is involved in building a model for aquaculture that does not harm ecosystems and provides a reliable source of healthy seafood. This book features contributions from renowned international authors, presenting high quality scientific chapters addressing key issues for effective health management of cultured aquatic animals. Available for open internet access, this book is an effort to reach the broadest diffusion of knowledge useful for both academic and productive sector.

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