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ORIGINAL PAPER

Evaluation of the probiotics *Bacillus subtilis* and *Lactobacillus plantarum* bioencapsulated in *Artemia* nauplii against vibriosis in European sea bass larvae (*Dicentrarchus labrax*, L.)

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Abstract Two potential probiotics *Bacillus subtilis* and *Lactobacillus plantarum* were evaluated for use in aquaculture as preventive measures against vibriosis. In vitro evaluation of the probiotics using co-culture assays with the pathogen *Vibrio anguillarum* and testing for the production of antibacterial substances showed the presence of antagonism and confirmed the production of antibacterial substances. Both potential probiotics were administered to the live fish feed *Artemia franciscana* nauplii, offering protection against a subsequent challenge of the nauplii with the fish pathogen *V. anguillarum*, with best survival rates of the nauplii and the most efficient protection offered by *B. subtilis*. Nauplii enriched with *B. subtilis* were further used to evaluate the protection of sea bass larvae against vibriosis. The untreated group of fish challenged with *V. anguillarum* presented low survival of 36.7 %, while the fish treated with nauplii enriched with the probiotic *B. subtilis* showed significantly increased survival rates of 86.7 % after challenge with the pathogen. The survival of healthy unchallenged fish treated with the probiotic was not significantly different from control unchallenged fish (90–94 %). Our results indicate that *B. subtilis* is a probiotic suitable to be used for the prevention of vibriosis in fish larvae and can be safely administered through their live feed *Artemia* nauplii.

Keywords Bioencapsulation · Probiotics · *Artemia franciscana* · *Dicentrarchus labrax* larvae · Vibriosis

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Introduction

Intensive culture of fish entails loss of a large number of animals due to bacterial infections, despite the fact that care is taken to ensure optimal nutritional and environmental factors (Trust 1986; Alderman 1988; Rigos and Troisi 2005). In euryaline fish farming the most prominent pathogenic bacteria are *Vibrio* (*Listonella*) *anguillarum* and *Photobacterium damsella* subsp. *piscicida* (Zorilla et al. 2003; Toranzo et al. 2005). The treatment of the bacterial diseases of fish especially in larval stages consists in the application of antibiotics either by bath (Samuelsen 2003), or using the live feed *Artemia* as a carrier of the antibiotics (Duis et al. 1995; Touraki et al. 1996, 1999). Although the application of antimicrobials has well documented beneficial effects to the infected animals, it might present an environmental hazard due to the development of microbial resistance of pathogens (Spangaard et al. 1993; Samuelsen et al. 1994; Rigos and Troisi 2005). Since strict food safety and quality requirements are required to increase food safety, the use of antibiotics is restricted and preventative measures are recommended against the introduction of pathogens to aquatic animal health, also being considered more cost effective than cure (FAO 2010). The most promising preventive method to control potential pathogens in fish culture is the use of probiotics (Kesarcodi-Watson et al. 2008) although the direct use of a probiotic by addition in the water column, presents great environmental consideration, due to the risk of microorganism pollution (Lara-Flores 2011). According to the broadened definition of Gram et al. (1999), probiotics are “live microbial supplements that beneficially affect the host animal by improving its microbial balance”. Various probiotics have been extensively used over the recent years to improve growth or survival of fish (Carnevali et al. 2006;

Makridis et al. 2008; Fjellheim et al. 2010), as immunomodulators (Salinas et al. 2005; Picchietti et al. 2007; Tapia-Paniagua et al. 2011), or to protect fish from vibriosis (Gildberg et al. 1997; Planas et al. 2006; Sorroza et al. 2012). However data on the effect of *Bacillus subtilis* and *Lactobacillus plantarum* on *V. anguillarum* are scarce and their suitability as probiotics to be used to protect fish larvae requires clarification. The administration of probiotics to target animals, namely fish larvae, using the technique of bioencapsulation of the probiotic in the live fish feed *Artemia* has been reported to be an interesting but scarcely studied approach (Gomez-Gil et al. 2000; Hai et al. 2010). Selected bacterial strains have been reported to protect *Artemia* from pathogens (Verschuere et al. 2000), while recently the incorporation of the probiotics *B. subtilis* and *B. licheniformis* in adults of *Artemia urniana* led to modulation of their gut microbiota (Motlagh et al. 2012).

The present study focused on the effect of two potential probiotics *B. subtilis* and *L. plantarum* to *Artemia* nauplii in terms of survival and protection from a challenge with *V. anguillarum*, and the efficacy of *Artemia* nauplii enriched with the selected probiotic to protect European sea bass larvae from vibriosis.

Materials and methods

Bacterial strains and culture conditions

Microbial strains originated from the Collection Espanola de Cultivos Tipo (CECT) and the National Collection of Industrial Bacteria (NCIMB, Aberdeen, Scotland). The strains that were used were *Bacillus subtilis* NCIMB 3610 and *L. plantarum* CECT 220. Stock cultures were maintained at −80 °C in 20 % glycerol, for long term storage. Bacteria were grown in MRS or NB broth at 30 °C, for 24 h (Papagianni et al. 2006). Optical density of cultures was monitored at 600 nm where maximum absorbance was observed. The Greek strain of the pathogen *Vibrio* (*Listonella*) *anguillarum*, 332A serotype O2, used for challenge experiments was grown in Brain Heart Infusion agar containing 2 % NaCl, at 22–24 °C, for 24 h (Magarinos et al. 1992).

Growth inhibition in co-culture assays

Both candidate probiotic strains were tested for antagonistic effects against the fish pathogen using broth co-culture assays. Two series were performed for each probiotic, one adding 10^4 cells of either *B. subtilis* or *L. plantarum* to 1 ml of BHI broth containing 2 % NaCl and a second by adding 10^7 probiotic cells. The original cell count of the pathogen *V. anguillarum* 332A was the same amounting to 10^4 cells. Mono cultures of each probiotic and of the pathogen acted as controls. An average CFU ml^{-1} was calculated by counting in each case and plotted against OD_{600}. Growth of probiotics and the pathogen was daily monitored at 600 nm and by spreading the co-culture on Brain Heart Infusion agar containing 2 % NaCl, morphological identification of each bacterium and counting. All cultures were performed in triplicate.

Production of antibacterial substances by the probiotics

Two series of experiments were performed one for each probiotic. Aliquots of fresh cultures of each candidate probiotic were inoculated in Nutrient broth at an initial cell density of 10^7 CFU ml^{-1} and incubated at 30 °C. Samples of 1 ml were withdrawn at 0, 24, 48 and 96 h, centrifuged at 200 × g and the supernatants sterilized through 0.45 μm pore -size filters and stored at 4 °C. The inhibitory activity of each supernatant was tested by the method of Gram et al. (1999). Briefly 450 μl of supernatant were added in tubes containing 500 μl Nutrient broth with 2 % NaCl and these were inoculated with 50 μl of *V. anguillarum*, yielding approximately 10^4 CFU ml^{-1} of the pathogen. As controls served samples in which the pathogen was inoculated in 950 μl of BHI broth. The three replicates used for each combination were incubated in a shaker (220 rotations/min, 30 °C) and the pathogen growth was monitored by optical density recordings of 100 μl aliquots at 600 nm in a Microplate Autoreader photometer (Bio-Tek Instruments), at 0, 24, 48, 72 and 120 h.

Administration of probiotics to *Artemia* nauplii

Experiments were performed on *A. franciscana* (Kellog) cysts (E.G. grade, Great Salt Lake strain, batch 11.119.03) kindly provided by INVE (Aquaculture Artemia Systems, Baasrode, Belgium) and stored at 4 °C. Hydration and decapsulation of the cysts in hypochlorite solution were performed as previously described (Sorgeloos et al. 1986), resulting in bacteria-free cysts. The cysts were re-suspended in a vessel containing 500 ml of filtered and autoclaved artificial seawater (35 g l^{-1}, pH 8.7) and they were allowed to hatch for 24 h under continuous filtered aeration and illumination (2,000 lux) at 28 ± 1 °C (Soltanian et al. 2007). At the end of the incubation period nauplii instar I were collected aseptically and used for enrichment according to the method of Patra and Mohamed (2003). Briefly, nauplii were thoroughly rinsed with filtered and autoclaved artificial seawater, transferred to vessels containing 500 ml sterile seawater at a density of 15 nauplii ml^{-1} and the appropriate probiotic or its culture cell free supernatant was administered to the nauplii.

Three treatments were performed for each candidate probiotic. In the first treatment with *B. subtilis*, a probiotic culture of 3×10^9 CFU ml^{-1} was used and 75 μl were administered per dose to the nauplii daily, 2 doses per day for a total of 2 days corresponding to the administration of a total of 1.8×10^6 CFU ml^{-1} of *Artemia* culture medium, after four doses to the nauplii over a period of 48 h. In the second treatment 150 μl of a *B. subtilis* culture of 7×10^9 CFU ml^{-1} were administered to the nauplii corresponding to 8.4×10^6 CFU ml^{-1} of *Artemia* culture medium after the total of four administered doses. In the third treatment the cell-free *B. subtilis* culture supernatant, after sterilization, was added in the sterile artificial seawater used for the culture of the nauplii at a concentration of 50 % (v/v).

In the first treatment with *L. plantarum* a probiotic culture of 2.6×10^9 CFU ml^{-1} was used and 75 μl were administered per dose to the nauplii daily, 2 doses per day for a total of 2 days corresponding to the administration of 1.5×10^6 CFU ml^{-1} of *Artemia* culture medium after four doses to the nauplii over a period of 48 h. In the second treatment 150 μl of a *L. plantarum* culture of 7.1×10^9 CFU ml^{-1} were administered to the nauplii corresponding to 8.4×10^6 CFU ml^{-1} of *Artemia* culture medium after the total of four doses. In the third treatment the cell-free *L. plantarum* culture supernatant, after sterilization, was added in the sterile artificial seawater used for the culture of the nauplii at a concentration of 50 % (v/v).

All experiments were performed in triplicate an as control served nauplii that did not receive any treatment with either candidate probiotic. Survival of *Artemia* nauplii was monitored for 48 h after the addition of the probiotics. The presence of the probiotic in the nauplii was confirmed at the end of each experiment after thorough washing of the nauplii with sterile seawater, homogenization and inoculation on plates with Nutrient agar, BHI agar containing 2 % NaCl and McConkey agar and incubation at 25 °C for 24–72 h.

Challenge of *Artemia* nauplii enriched with probiotics with *V. anguillarum*

Challenge of *Artemia* nauplii was performed according to the method used by Defoirdt et al. (2005). Briefly, cells from of a *V. anguillarum* culture (1×10^9 CFU ml^{-1}) were washed twice with sterile saline and 200 μl were used to challenge the nauplii. This corresponds to a bacterial density of 2×10^5 CFU ml^{-1} of *Artemia* culture medium. Nauplii that were not treated with any probiotic nor challenged served as negative control while nauplii that were not fed with probiotics but were challenged with the pathogen were the positive control. All experiments were performed in triplicate and *Artemia* survival was monitored for a total of 96 h. The presence of the probiotic or the pathogen in the nauplii was confirmed at the end of each experiment after thorough washing of the nauplii with sterile seawater, homogenization and inoculation on plates with Nutrient agar, BHI agar containing 2 % NaCl and McConkey agar and incubation at 25 °C for 24–72 h.

Administration of *Artemia* nauplii enriched with the probiotic *B. subtilis* to *D. labrax* larvae to evaluate harmlessness of probiotic

Sea bass larvae, 50 days old, (of an average weight of 80 mg, length 1.3–1.9 cm approximately), kindly provided by a commercial local hatchery, were kept in filtered artificial seawater under continuous aeration at 20 °C, with a photoperiod of 12 h. Prior to any experiments a 10-day conditioning period was allowed. Fish were fed twice daily using *A. franciscana* nauplii at a level of 5–10 nauplii ml^{-1} seawater. Fish density never exceeded 5 individuals per liter and about one-third of the tank water was renewed daily. After the acclimatization period, groups of 20 fish were thoroughly rinsed and transferred in clean artificial seawater at separate aquaria of a capacity of 10 l each.

Since in the experiments with both probiotics on *Artemia* nauplii, *B. subtilis* resulted in better survival rates of the nauplii with or without challenge with the pathogen, this probiotic was chosen for further experiments. To examine the harmlessness of probiotic on sea bass larvae, fish were fed with nauplii enriched with the *B. subtilis* for 5 consecutive days and their survival was recorded for a total of 15 days. *Artemia* nauplii enriched with four doses of 150 μl of a *B. subtilis* culture of 7×10^9 CFU ml^{-1}, as described above, after being thoroughly washed with autoclaved and filtered artificial seawater they were administered to fish larvae at 2 doses of nauplii daily (one every 12 h) at a density of 750 nauplii per individual per dose according to Touraki and Niopas (2012). Following the 5 days period, fish were fed with *Artemia* nauplii without any probiotic. As control served fish that were fed throughout the experiment with *Artemia* nauplii that received no probiotic treatment. Experiments were conducted in triplicate and survival was recorded daily for a total of 15 days. Fish samples were microbiologically tested on BHI agar for the presence of the inoculated strain.

Challenge of fish larvae with *V. anguillarum* and evaluation of treatment with the probiotic *B. subtilis*

Sea bass larvae of an average body weight of 90 mg were with *Artemia* nauplii enriched with *B. subtilis* for 5 consecutive days, twice a day, as described above. All

experiments were conducted in triplicate, with 20 fish per experimental tank. A negative control group was used in which fish were neither fed with the probiotic nor challenged with the pathogen as well as a positive control that was challenged with the pathogen but did not receive any probiotic treatment. The challenge with *V. anguillarum 332A* (1.6×10^8 CFU ml^{-1}) was performed in baths for 8 h at 21–24 °C as recommended by Sorroza et al. (2012). Survival of fish was recorded daily and each moribund or dead fish was removed from the tank and further analyzed for the presence of the pathogen.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey test in case the values showed significant differences. Statistical significance was set at a level of 0.05. The survival curves of sea bass larvae were estimated using survival analysis by the Kaplan–Meier method followed by curve comparison by long-rank test and Gehan–Breslow–Wilcoxon test.

Results

Growth inhibition in co-culture assays

The two potential probiotic strains were tested for antagonistic effects on the growth of *V. anguillarum* in broth co-culture assays with the pathogen. After 96 h in co-culture *B. subtilis* inhibited the growth of the pathogen by 33 % when added at the low concentration of 8.2×10^4 CFU ml^{-1} and this inhibition increased to 42 % when the concentration of the probiotic was 1.1×10^7 CFU ml^{-1} (Fig. 1a, b). In the case of *L. plantarum*, the inhibition of growth of the pathogen amounted to 16 % at the low concentration of the probiotic and to 21 % at the higher concentration of the probiotic. (Fig. 1c, d). It should be noted that in the case of *B. subtilis* the inhibition of growth was gradual and the growth of the pathogen declined from 0 to 96 h of co-culture, without however complete inhibition of its growth. To the contrary when *L. plantarum* was used, the growth of the pathogen was completely inhibited at 24 and 48 h of co-culture of the pathogen with the high

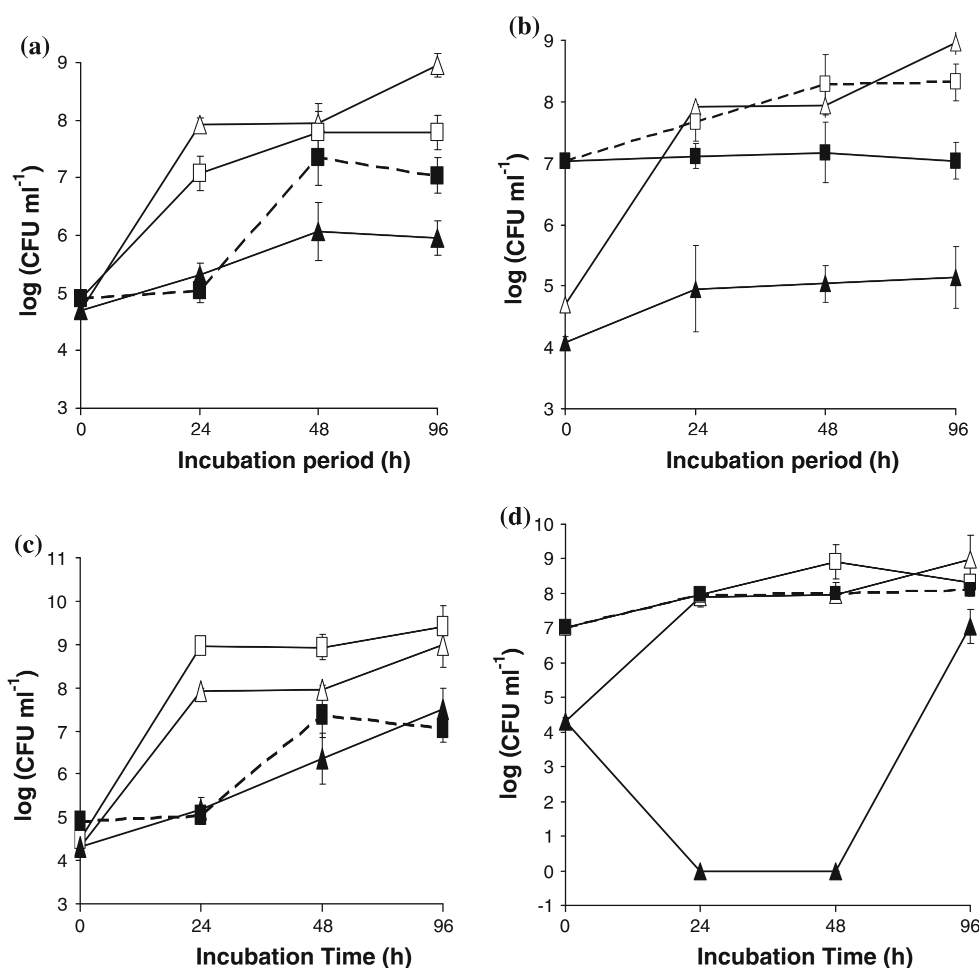

Fig. 1 Growth inhibition in co-culture assays of *V. anguillarum* with: **a** *B. subtilis* at 10^4 CFU ml^{-1}, **b** *B. subtilis* at 10^7 CFU ml^{-1}, **c** *L. plantarum* at 10^4 CFU ml^{-1} and **d** *L. plantarum* at 10^7 CFU ml^{-1}. *Open triangle* growth of *V. anguillarum* alone, *open square* growth of probiotic alone, *filled triangle* growth of *V. anguillarum* in the presence of the probiotic, *filled square* growth of the probiotic in the presence of Vibrio

concentration of the probiotic, rising again after 96 h of co-culture at levels significantly lower from control ($P < 0.001$).

Production of antibacterial substances by the probiotics

In order to screen whether the inhibition of the pathogen was due to antagonism or to antibacterial substances produced by the potential probiotics, the pathogen was cultured in cell-free supernatants of the two probiotics. Growth inhibition of the pathogen was significant in the 48 and 72 h supernatants of *B. subtilis* ($P < 0.001$), while in the other supernatants of the probiotic tested there was no significant reduction of the growth of the pathogen ($P > 0.05$) (Fig. 2a). The inhibition of growth was however more evident in the case of culture of the pathogen in the 48 and 72 h supernatants of *L. plantarum* and complete inhibition of the pathogen was observed after 24 h of culture (Fig. 2b).

Administration of probiotics to *Artemia* nauplii

Artemia survival was reduced due to the administration of probiotics (Fig. 3a, b). In the case of *B. subtilis*, although survival rates were slightly lower at both concentrations of the probiotic (Fig. 3a), they were not significantly different compared to the control values ($P > 0.05$). However, in the *L. plantarum* series, survival appeared significantly reduced compared to control ($P < 0.05$) reaching 58 % in the higher concentrations of 10^9 CFU ml^{-1} of the probiotic (Fig. 3b). The cell free supernatant of both probiotics was highly toxic to *Artemia* nauplii, leading to zero survival rates at 48 h after the administration.

Challenge of *Artemia* nauplii enriched with probiotics with *V. anguillarum*

In order to evaluate any possible protection offered to *Artemia* by the probiotics, the nauplii were challenged following the administration of the probiotics (Fig. 4). Our results showed a marked protection of the nauplii due to probiotics, which was higher when the higher concentrations of probiotics were used. In terms of survival, nauplii that were not treated with either probiotic showed survival rates of 33 %. Treatment with the high concentration of 10^9 CFU ml^{-1} of *L. plantarum* resulted in improved survival rates of 84 %, a value however significantly different from the survival of 95 % of control nauplii that were not challenged ($P < 0.001$). A better protection of *Artemia* nauplii from the pathogen appeared to be offered by *B. subtilis*, with survival amounting to 58 % at the low dose of the probiotic and to 99 % at the high dose of the probiotic, a value not significantly different ($P > 0.05$) from the survival of the control nauplii that received no challenge.

Administration of *Artemia* nauplii enriched with the probiotic *B. subtilis* to *D. labrax* larvae to evaluate harmlessness of probiotic

Since best survival rates of *Artemia* nauplii and best protection of them against a *V. anguillarum* infection was

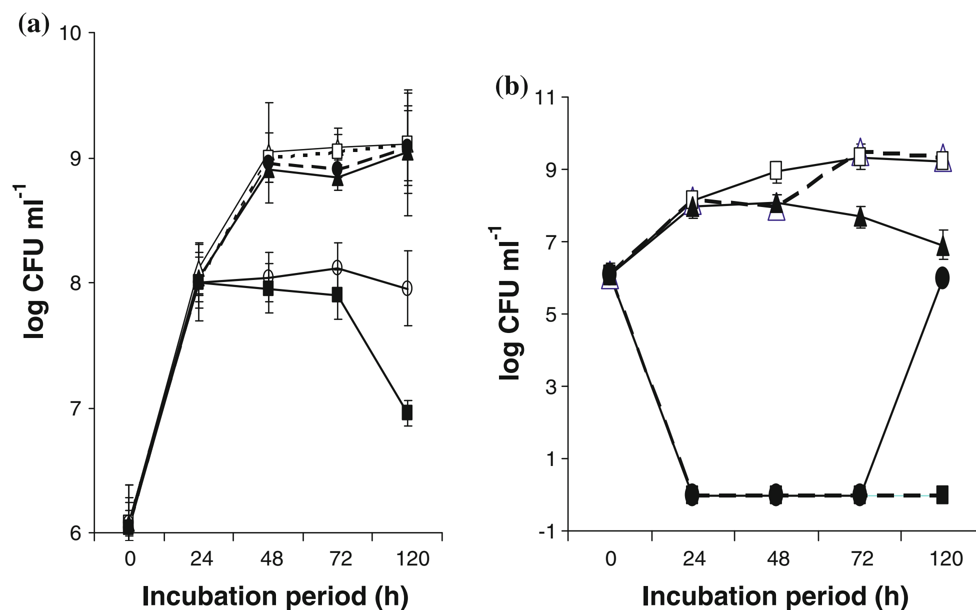

Fig. 2 Growth *curves* of *V. anguillarum* cultured in the presence of cell free supernatants of **a** *B. subtilis* and **b** *L. plantarum* to screen for the production of antibacterial substances by the probiotics. *Open triangle* control Vibrio with no S/N, *open square* culture of pathogen in S/N 0 h, *filled triangle* culture of pathogen in S/N 24 h, *open circle* culture of pathogen in S/N 48 h, *filled square* culture of pathogen in S/N 72 h and *filled circle* culture of pathogen in S/N 96 h

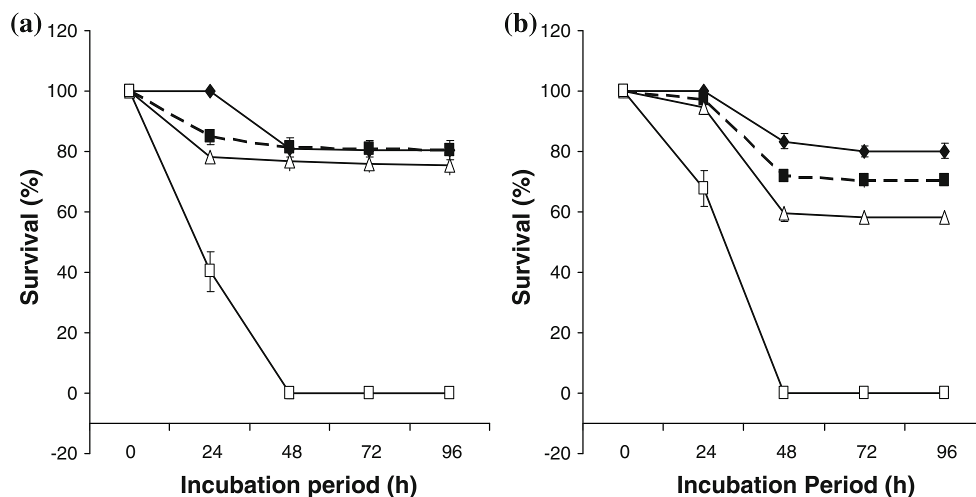

Fig. 3 Effect of the administration of *B. subtilis* (**a**) and *L. plantarum* (**b**) on the survival of *Artemia* nauplii. *Diamond* control survival of *Artemia* nauplii without any probiotic, *filled square* survival of *Artemia* following administration of 10^8 CFU ml^{-1} probiotics, *open triangle* survival of *Artemia* following administration of 10^9 CFU ml^{-1} probiotics, *open square* survival of *Artemia* following administration of cell free supernatant of probiotic culture

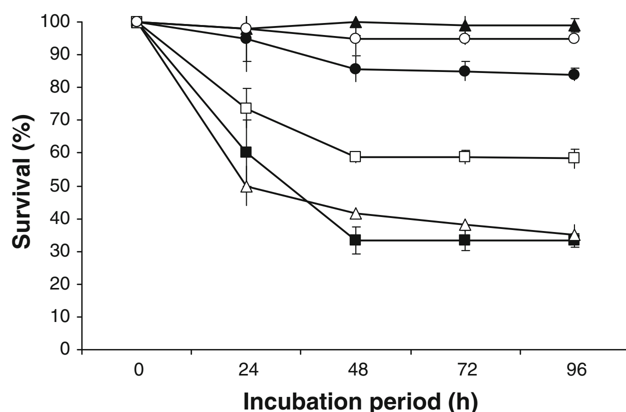

Fig. 4 Effect of the administration of probiotics to *Artemia* nauplii following challenge with *V. anguillarum*. *Filled square* positive control group challenged with pathogen without any probiotic treatment, *open square* group receiving low dose of 10^8 CFU ml^{-1} *B. subtilis* prior to challenge, *filled triangle* group receiving high dose of 10^9 CFU ml^{-1} *B. subtilis* prior to challenge, *open triangle* group receiving low dose of 10^8 CFU ml^{-1} *L. plantarum* prior to challenge, *filled circle* group receiving high dose of 10^9 CFU ml^{-1} *L. plantarum* prior to challenge and *open circle* negative control group, no challenge, no probiotic treatment

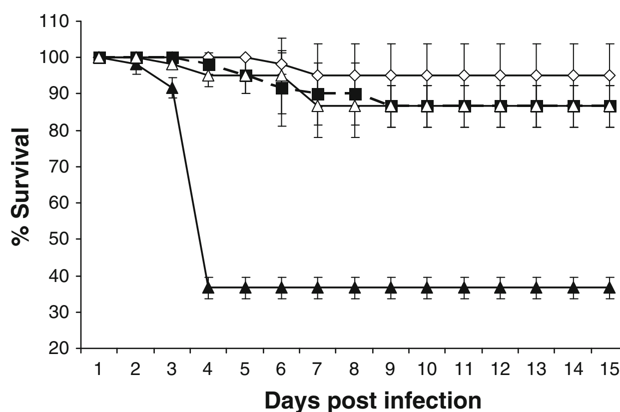

Fig. 5 Survival of *D. labrax* larvae fed with *B. subtilis* enriched *Artemia* nauplii and challenged with *V. anguillarum*. *Open circle* negative control group of fish that did not receive probiotic treatment or challenge, *filled square* fish that were fed with *Artemia* nauplii enriched with *B. subtilis* but were not challenged, *filled triangle* positive control group of fish that did not receive probiotic treatment and were challenged with *V. anguillarum*, (*open triangle*) group of fish that was fed with *Artemia* nauplii enriched with *B. subtilis* prior to challenge with the pathogen

observed using *B. subtilis*, this probiotic was chosen to be administrated to fish larvae. Evaluation of the possible harmful effects of the probiotic to fish by the administration of *Artemia* nauplii enriched with the probiotic showed no harmful effects (Fig. 5), since survival (86 % ± 5.7) was not significantly different ($P > 0.05$) from control (95 % ± 8.6). Comparison of the survival curves showed absence of any significant difference between control and *B. subtilis* treated sea bass larvae (χ^2 3.38, *P* value 0.066 at Log-Rank test).

Challenge of fish larvae with *V. anguillarum* and evaluation of treatment with the probiotic *B. subtilis*

In the challenge experiment, the mortality of the positive control, that is untreated challenged fish, amounted to 54 %, the mortality of the negative control to 5 %. The mortality of the probiotic treated sea bass larvae after challenge amounted to 14 % and was significantly reduced in comparison to the negative control group (Fig. 5). Comparison of the survival curves showed significant

difference among the *B. subtilis* treated—challenged fish and the positive control group (χ^2 59.22, *P* value <0.001). Fish that were affected showed signs of vibriosis, mainly consisting of hemorrhagic septicaemia and the pathogen was recovered from the dead fish. Therefore fish mortalities were attributed to the pathogen.

Discussion

The extensive use of antibiotics to treat fish infections in aquaculture has led to the necessity of finding novel alternative methods, rather prophylactic treatments instead of therapeutic. As such, the use of probiotics has been suggested only a few years ago (Gomez-Gil et al. 2000; Burr and Gatlin 2005). Recently probiotics have been successfully used either to promote growth of fish (Carnevali et al. 2006), or to induce immunological responses (Picchietti et al. 2007, Makridis et al. 2008, Tapia-Paniagua et al. 2011). It has recently been reported (Swain et al. 2009) that the probiotics *Streptococcus phocae* and *Enterococcus faecium* control vibriosis in the shrimp *Penaeus monodon*. Protection of fish from infections has been reported using a variety of probiotics such as *Pseudomonas fluorescens* (Gram et al. 1999), *Roseobacter* (Planas et al. 2006), *Vagococcus fluvialis* (Sorroza et al. 2012), *Salvenilus fontinalis* (Boutin et al. 2011) and mixtures of *Lactobacillus* bacteria (Talpur et al. 2011). However, the risk of microbiological pollution of the environment when the probiotics are added in the water (Lara-Flores 2011) and the short survival of probiotics in seawater (Gatesoupe 2008) have been reported as limiting factors in their use in aquaculture. In the present study an alternative approach of the incorporation of probiotics in the live fish feed *Artemia* nauplii is used. Our results showed that *B. subtilis* and *L. plantarum* protect *Artemia* nauplii against *V. anguillarum* and in addition the probiotic *B. subtilis* protects sea bass larvae from *V. anguillarum* infection when administered through the nauplii of *Artemia*.

The growth of the pathogen was successfully inhibited by both probiotics in co-culture as well as when the pathogen was incubated in cell free supernatants of the probiotics. Similar inhibition of growth has been reported for *V. harveyi* in co-culture experiments with *B. subtilis* BT23 (Vaseeharan and Ramasamy 2003; Banerjee et al. 2007) as well as in co-culture with *L. plantarum* (Kongnum and Hongpattarakere 2012) and these probiotics have been suggested respectively for use as probiotic treatments of the black tiger shrimp *Penaeus monodon* and the white shrimp *Litopenaeus vanamei*. The recovery of *V. anguillarum* after 96 h of culture with the high dose of *L. plantarum* in combination to the slight decrease in growth of the probiotic observed in our study, might be attributed to competition for nutrients. Growth of lactic acid bacteria is inhibited in nutrient exhaustion conditions (Leroy and De Vuyst 2001) while *V. anguillarum* has been reported to survive long term starvation (Nelson et al. 1997). However, the fact that inhibition of growth of the pathogen was evident in our study when the pathogen was cultured in the 48 and 72 h cell free supernatants of the probiotics, suggests that inhibition might be attributed to the production of bacteriocins by the probiotics.

The administration of probiotics to *Artemia* nauplii led to slightly decreased survival rates when high concentrations of the probiotics were used and this decrease in survival was significant ($P < 0.05$) in the *L. plantarum* treated group. The higher survival rates of *Artemia* nauplii observed by other investigators (Patra and Mohamed 2003; Motlagh et al. 2012) are possibly due to the lower concentrations of probiotics used in these studies. The lower survival rates observed in our study are in agreement with the adverse effects of the administration of increased levels of *Bacillus* sp to angelfish (Farahi et al. 2011). In a study conducted by Dehghan et al. (2011) on the enrichment of *Artemia urniana* nauplii with *B. subtilis*, although survival of nauplii was not recorded, the authors concluded that *Artemia* nauplii are suitable to be used for bio-vaccination for the control and treatment of diseases, with time of enrichment being of crucial importance since it affects the levels of probiotics. The potency of the antibacterial substances produced by *B. subtilis* and *L. plantarum* is evident from the fact that zero survival rates were observed for nauplii in cell free supernatants of both probiotics. It appears that an optimal concentration of the appropriate probiotic is necessary to offer protection to *Artemia* and possibly fish against pathogens, without however showing any toxicity effects to the means of delivery, namely the nauplii. Selected bacterial strains have been reported to protect *Artemia* against the pathogen *V. proteolyticus* only when living cells or the probiotics were present (Verschuere et al. 2000). In our study *B. subtilis* administered at 10^9 CFU ml^{-1} of culture, offered the best protection to *Artemia* in a challenge with *V. anguillarum*, since the survival of the nauplii after challenge was not significantly different than the negative control. Hence *B. subtilis* was selected for further experiments with sea bass larvae. This strain was harmless to sea bass larvae since no mortality was observed 10 days after the administration of *Artemia* nauplii enriched with *B. subtilis* for a 5 day period and the comparison of the survival curves of the probiotic treated and the control group of fish showed no significant difference. In the experimental challenge however performed to evaluate the protection offered by this probiotic against infection by *V. anguillarum*, mortality appeared greatly reduced in the *B. subtilis* treated fish compared to the

positive control group. A similar reduction in mortality was reported for rainbow trout after the administration of the probiotic *Pseudomonas fluorescens* in the water and following a challenge with *V. anguillarum* (Gram et al. 1999). In addition Sorroza et al. (2012) observed protection of the European sea bass following the administration of the probiotic *Vagococcus fluvialis* by means of a commercial dry fish feed. However there is no report on the ability of probiotics to offer protection to fish larvae against pathogens, or studies employing the administration of the probiotics through the live fish feed *Artemia* in challenge tests. The positive immunostimulatory effect of probiotics administered to sea bream larvae through the live fish feed *Artemia* was reported by Picchietti et al. (2007), although no challenge test was performed. Since an environmentally friendly method is to deliver the probiotics to fish is necessary to avoid microbial pollution (Lara-Flores 2011) and fish larvae feed on *Artemia* nauplii, the live fish feed as route of administration was considered an interesting but scarcely studied approach (Gomez-Gil et al. 2000). Recently Vine et al. (2006) suggested that an optimization of the loading of probiotic bacteria to live food is required. The results in our study present evidence for the ability of *Artemia* nauplii to act as carriers of the probiotic *B. subtilis* to sea bass larvae, offering protection against vibriosis.

Conclusion

In conclusion, our data provide strong evidence that *B. subtilis* could be used as probiotic bacteria administered through the live fish feed *Artemia* nauplii, to protect sea bass larvae against infection by *V. anguillarum* providing an environmentally friendly and antibiotic-free alternative method for the prophylaxis of marine fish larvae of vibriosis.

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References

Alderman DJ (1988) Fisheries chemotherapy: a review. In: Muir JF, Roberts KJ (eds) Recent advances in aquaculture, vol 3. Croom Helm, London. United Kingdom and Timber Press, Portland, pp 1–61

Banerjee S, Devaraja TN, Shariff M, Yusoff FM (2007) Comparison of four antibiotics with indigenous marine *Bacillus* spp. in controlling pathogenic bacteria from shrimp and *Artemia*. J Fish Dis 30:383–389

Boutin S, Bernatchez L, Audet C, Deromea N (2011) Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*. Vet Microbiol. doi:10.1016/j.vetmic.2011.09.002

Burr G, Gatlin D (2005) Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. J World Aquac Soc 36:425–436

Carnevali O, De Vivo L, Sulpizio R, Gioacchini G, Olivotto I, Silvi S, Cresci A (2006) Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.) with particular attention to IGF-1, myostatin and cortisol gene expression. Aquaculture 258:430–438

Defoirdt T, Bossier P, Sorgeloos P, Verstraete W (2005) The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. Environ Microbiol 7(8):1239–1247

Dehghan M, Jafariyan H, Rezai MH, Amoozagar MA, Sahandi J (2011) Potential of the brine shrimp (*Artemia urniana*) enrichment with two species of Bacillus and Yeast (*Saccharomyces cerevisiae*). World J Fish Mar Sci 3(6):523–528

Duis K, Hammer C, Beveridge MCM, Inglis V, Braum E (1995) Delivery of quinolone antibacterials to turbot, *Scophthalmus maximus*, L. via bioencapsulation: quantification and efficacy trial. J Fish Dis 18:229–238

FAO (2010) Aquaculture planning policy formulation and implementation for sustainable development by Cicile Brugere, Neil Ridler, Graham Haylor, Graeme Macfadyen, Nathanael Hishamunda, Food And Agriculture Organization Of The United Nations Rome, Fao Fisheries And Aquaculture Technical Paper

Farahi A, Kasiri M, Alamshahi F (2011) The effects on growth, survival and tolerance against environmental stressor (high temperature) of different concentrations probiotic Bacillus sp., fed to angelfish (*Pterophyllum scalare* Schultze, 1823) larvae. J Anim Vet Adv 10(17):2305–2311

Fjellheim AJ, Klingenberg G, Skjermo J, Aasen IM, Vadstein O (2010) Selection of candidate probionts by two different screening strategies from Atlantic cod (*Gadus morhua* L.) larvae. Vet Microbiol 144:153–159

Gatesoupe FJ (2008) Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J Mol Microbiol Biotechnol 14(1–3):107–114

Gildberg A, Mikkelsen H, Sandaker E, Ringo E (1997) Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). Hydrobiologia 352:279–285

Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270

Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl Environ Microbiol 65:969–973

Hai NV, Buller N, Fotedar R (2010) Encapsulation capacity of *Artemia* nauplii with customized probiotics for use in cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye, 1986). Aquac Res 41:893–903

Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture 274:1–14

Kongnum K, Hongpattarakere T (2012) Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*. Fish Shellfish Immunol 32(1):170–177

Lara-Flores M (2011) The use of probiotic in aquaculture: an overview. Int Res J Microbiol 2(12):471–478

Leroy F, De Vuyst L (2001) Growth of the bacteriocin-producing *Lactobacillus sakei* strain CTC 494 in MRS broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for growth of lactic acid bacteria. Appl Environ Microbiol 67(10):4407–4413

Magarinos B, Romalde JL, Bandín I, Fouz B, Toranzo AE (1992) Phenotypic, antigenic and molecular characterization of *Pasteurella piscicida* strains isolated from fish. Appl Environ Microbiol 58:3316–3322

Makridis P, Martins S, Reis J, Dinis MT (2008) Use of probiotic bacteria in the rearing of Senegalese sole (*Solea senegalensis*) larvae. Aquacult Res 39:627–634

Motlagh HRA, Farhangi M, Rafiee G, Noori F (2012) Modulating gut microbiota and digestive enzyme activities of *Artemia* urniana by administration of different levels of *Bacillus subtilis* and *Bacillus licheniformis*. Aquac Int doi:10.1007/s10499-012-9497-5

Nelson DR, Sadlowski Y, Eguchi M, Kjelleberg S (1997) The starvation-stress response of *Vibrio* (*Listonella*) *anguillarum*. Microbiology 143:2305–2312

Papagianni M, Avramidis N, Filioussis G, Dasiou D, Ambrosiadis I (2006) Determination of bacteriocin activity with bioassays carried out on solid and liquid substrates: assessing the factor "indicator microorganism". Microb Cell Fact 10(5):30–54

Patra SK, Mohamed KS (2003) Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. Aquac Int 11:505–514

Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, Carnevali O, Cresci A, Abelli L (2007) Effects of administration of probiotic strains on GALT of larval gilthead sea bream: immunohistochemical and ultrastructural studies. Fish Shellfish Immunol 22(1–2):57–67

Planas M, Peréz-Lorenzo M, Hjelm M, Gram L, Fiksdal IU (2006) Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. Aquaculture 255:323–333

Rigos G, Troisi GM (2005) Antibacterial agents in mediterranean finfish farming: a synopsis of drug pharmacokinetics in important euryhaline fish species and possible environmental implications. Rev Fish Biol Fish 15:53–73

Salinas I, Cuesta A, Esteban MA, Meseguer J (2005) Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead sea bream cellular innate immune responses. Fish Shellfish Immunol 19:67–77

Samuelsen OB (2003) Administration of the antibacterial agents flumequine and oxolinic acid to small turbot (*Scophthalmus maximus L.*) by bath. J Appl Ichthyol 19:55–58

Samuelsen OB, Lunestad BT, Ervik A, Fjelde S (1994) Stability of antimicrobial agents in artificial marine sediment studied under laboratory conditions. Aquaculture 126:283–290

Soltanian S, Dhont J, Sorgeloos P, Bossier P (2007) Anti-infectious potential of beta-mercapto-ethanol treated baker's yeast in gnotobiotic *Artemia* challenge test. J Appl Microbiol 104:1137–1146. doi:10.1111/j.1365-2672.2007.03629.x

Sorgeloos P, Lavens P, Leger P, Tackaert W, Versichele D (1986) Manual for the culture and use of the brine shrimp *Artemia* in aquaculture. Artemia Reference Center, Faculty of Agriculture, State University of Ghent, Belgium, pp 91–95

Sorroza L, Padilla D, Acosta F, Román L, Grasso V, Vega J, Real F (2012) Characterization of the probiotic strain *Vagococcus fluvialis* in the protection of European sea bass (*Dicentrarchus labrax*) against vibriosis by *Vibrio anguillarum*. Vet Microbiol 155(2–4):369–373

Spangaard B, Jorgensen F, Gram L, Huss HH (1993) Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture 115:195–207

Swain SM, Singh C, Arul V (2009) Inhibitory activity of probiotics *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 against vibriosis in shrimp *Penaeus monodon*. World J Microbiol Biotechnol 25:697–703

Talpur AD, Memon AJ, Khan MI, Ikhwanuddin M, Danish Daniel MM, Abol-Munafi AB (2011) Supplementation of Indigenous Lactobacillus Bacteria in Live Prey and as Water Additive to Larviculture of *Portunus pelagicus* (Linnaeus, 1758). Adv J Food Sci Technol 3(5):390–398

Tapia-Paniagua ST, Reyes-Becerril M, Ascencio-Valle F, Esteban MÁ, Clavijo E (2011) Modulation of the Intestinal Microbiota and Immune System of Farmed *Sparus aurata* by the Administration of the Yeast *Debaryomyces hansenii* L2 in Conjunction with Inulin. J Aquac Res Develop S1:012. doi:10.4172/2155-9546.S1-012

Toranzo AE, Magarinos B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246:37–61

Touraki M, Niopas I (2012) Bioencapsulation and pharmacokinetic study of oxolinic acid in rotifers (*Brachionus plicatilis*), *Artemia franciscana* nauplii and metanauplii. Aquac Res. doi:10.1111/j.1365-2109.2011.03075.x

Touraki M, Mourelatos S, Karamanlidou G, Kalaitzopoulou S, Kastritsis C (1996) Bioencapsulation of chemotherapeutics in *Artemia* as a means of prevention and treatment of infectious diseases of marine fish fry. Aquac Eng 15(2):133–147

Touraki M, Niopas I, Kastritsis C (1999) Bioaccumulation of trimethoprim, sulfamethoxazole and *N*-acetyl-sulfamethoxazole in *Artemia* nauplii and residual kinetics in sea bass larvae after repeated oral dosing of medicated nauplii. Aquaculture 175:15–30

Trust TJ (1986) Pathogenesis of infectious diseases of fish. Ann Rev Microbiol 40:479–502

Vaseeharan B, Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36:83–87. doi:10.1046/j.1472-765X.2003.01255.x

Verschuere L, Heang H, Criel G, Sorgeloos P, Verstraete W (2000) Selected bacterial strains protect *Artemia* spp. from the pathogenic effects of *Vibrio proteolyticus* CW8T2. Appl Environ Microbiol 66(3):1139–1146

Vine NG, Leukes WD, Kaiser H (2006) Probiotics in marine larviculture. FEMS Microbiol Rev 30:404–427. doi:10.1111/j.1574-6976.2006.00017.x

Zorilla I, Arijo S, Chabrillon M, Diaz P, Martinez-Manzanares E, Balebona MC, Moriñigo MA (2003) *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup), and evaluation of the potential virulence role of their extracellular products. J Fish Dis 26:103–108