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Probiotics for shrimp larviculture: review of field data from Asia and Latin America

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Abstract

Disease problems have emerged as major constraints in aquaculture production. The prophylactic application of antibiotics is expensive and detrimental, i.e. selection of bacteria that are drug-resistant or more virulent and the prevalence of drug residues in reared animals. Probiotics, which compete with bacterial pathogens for nutrients and/or inhibit the growth of pathogens, could be a valid alternative to the prophylactic application of chemicals. A mixture of specific *Bacillus* strains was designed following a research programme on the ability of numerous *Bacillus* strains to inhibit a range of pathogenic *Vibrio* strains, to grow under conditions prevailing in shrimp hatcheries and to degrade waste products. These strains were then included in bioassays and challenge tests in order to confirm the lack of toxin production and pathogenicity to humans, target organisms and the environment. Here, we report on the performance of a commercially available mixture of *Bacillus* strains (SANOLIFE® MIC), using data from Asian and Latin-American hatcheries, with *Penaeus monodon* (Fabricius 1798) and *Litopenaeus vannamei* (Boone 1931). These results show that probiotics may be a suitable alternative to the prophylactic use of antibiotics. Obviously, minimizing the risk of vibriosis demands a multi-disciplinary approach, including good hygiene and sanitation measures to reduce the input of potential pathogens, as well as a suitable farm management.

Keywords: probiotic, *Bacillus*, *Vibrio*, larviculture, disease

Introduction

Major losses in penaeid hatcheries are due to general problems with water quality control and *Vibrio* spp., which are opportunistic bacteria. *Vibrio* spp. are normally present in the culture facilities, as well as in the larval gut flora and in the live feed and they usually cause disease under sub-optimal culture conditions. The application of antibiotics is expensive and detrimental, i.e. selection of bacteria that are drug-resistant or more virulent, prevalence of drug residues in reared animals (Moriarty 1999; Gilbert & McBain 2003; Holmström, Gräslund, Wahlström, Poungshompoo, Bengtsson & Kautsky 2003). Probiotics, which compete with bacterial pathogens for nutrients and/or inhibit the growth of pathogens, could be a valid alternative to the prophylactic application of chemicals, antibiotics and biocides (Moriarty 1999). In addition, the role of microorganisms in water quality improvement is well documented. Much doubt on the efficacy and safety of probiotics on the market comes from the use of ineffective bacterial species, unrealistic claims, lack of scientific evidence and poor quality control during production of the finished product, or inappropriate delivery methods leading to contamination or reduced performance (Temmerman, Huys, Pot & Swings 2003). The development of suitable probiotics is not a simple task and requires empirical and fundamental research, full-scale trials, as well as the development of appropriate monitoring tools and controlled production.

As Priest (1993) states: 'species such as *Bacillus licheniformis* (Weigmann 1898) and *B. subtilis* (Ehrenberg 1835) are universally distributed and dominate the marine flora to such an extent that they could be considered primary inhabitants of the oceans '. . .. *Bacillus* (Cohn 1872) generally occur more frequently in sediments than in the water column; at lower levels, *Bacillus* spores may account for up to 80% of the total heterotrophic flora'. There are many reports

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of *Bacillus* in marine sediments (e.g. Siefert, Larios-Sanz, Nakamura, Slepecky, Paul, Moore, Fox & Jurtshuk Jr 2000) and therefore they are naturally ingested by animals such as shrimps that feed in or on the sediment (e.g. Rengpipat, Phianphak, Piyatiratitivorakul & Menasveta 1998).

By following a scientific approach, we developed a line of *Bacillus* probiotics for shrimp larviculture and growout. Here, we report on the selection process and the performance of a commercially available mixture of *Bacillus* strains (SANOLIFE® MIC, INVE Aquaculture, Phichit, Thailand), using data from an Asian and Latin-American hatcheries, with *Penaeus monodon* (Fabricius, 1798) and *Litopenaeus vannamei* (Boone, 1931).

Materials and methods

Selection

Over 70 *Bacillus* strains were obtained from culture collections and commercial suppliers. Pathogenic *Vibrio* strains were either obtained from the culture collection from the University of Gent (BCCM/LMG Bacteria Collection), or directly isolated from the haemolymph of diseased animals. The *in vitro* inhibitory activity of the *Bacillus* strains against these *Vibrio* strains was evaluated by using a cross-streak method, on both marine agar and tryptone soya agar (Oxoid, Basingstoke, UK) amended with NaCl in order to reach a salinity of 25 g L^{-1}.

The growth rate of the best performing strains was evaluated by measuring the absorbance at 600 nm over a period of up to 24 h, of cultures inoculated in 1/2 strength tryptone soya broth, amended with NaCl in order to give a final salinity of 0, 10, 20, 30 or 40 g L^{-1}. In most cases, bacterial growth rates were fast and determinations were stopped after 8 h.

These best strains were then screened for the absence of *Bacillus cereus* enterotoxin (BCET–RPLA toxin detection kit, Oxoid) and included in challenge tests in order to confirm the lack of pathogenicity to the target organisms. SPF *L. vannamei* shrimp were challenged orally and by immersion with individual strains or mixtures of strains at concentrations 10–100 × higher than those to be encountered commercially. The shrimp were immersed for 3 h in a bacterial bath, with aeration supplied, and then transferred into the rearing system. They were kept in the aquaria for 7 days, being fed pellets (A2 monodon high-performance shrimp feed/shrimp complete grower, INVE Aquaculture, Dendermonde, Belgium) that had previously been soaked in a bacterial suspension.

Performance

Following various pilot-scale studies, the best-performing mixture of *Bacillus* strains (SANOLIFE® MIC) was evaluated under laboratory scale, pilot-scale conditions, and under commercial or pre-commercial conditions in various Asian and Latin-American countries. We will use data from a hatchery in Thailand (*P. monodon*) and those from a hatchery in Brazil (*L. vannamei*) as example.

In both hatcheries, the *Bacillus* spores present in the commercial product (together with required nutrients) were germinated before application in the rearing tanks by suspending the product in sterile water (drinking water or disinfected seawater) for up to 8 h. The appropriate amount of germinated *Bacillus* was immediately applied to the tanks in order to reach a final concentration of 1–5 × 10^4 CFU mL^{-1}. The probiotics were applied daily.

Thai hatchery

In three separate tests run over a period of 1 year, the performance of the *Bacillus* mixture was compared with antibiotic prophylactic and negative control. *P. monodon* nauplii were reared (4 × 175 L tanks per treatments in each test) until postlarvae (PL) 10, and fed a combination of *Chaetoceros*, *Artemia* nauplii and Lansy shrimp (40% live food substitution) INVE larval diet.

In the first of these tests, the concentration of *Vibrio* spp. was followed daily in each tank water using dip slides (BDS-G, INVE Aquaculture, Thailand).

Brazilian hatchery

The performance of the *Bacillus* mixture was compared with prophylactic antibiotic (in combination with formaldehyde treatment in the early PL stages) and negative control (without the addition of any chemicals). Each 20 MT tank was stocked with 5 million *L. vannamei* N3 per tank. The water disinfection procedure included chlorine addition, followed by neutralization with thiosulphate. Shrimp larvae were fed microalgae, *Artemia* and commercial larval diets. Additional information on the set-up of this hatchery was not disclosed by the hatchery manager.

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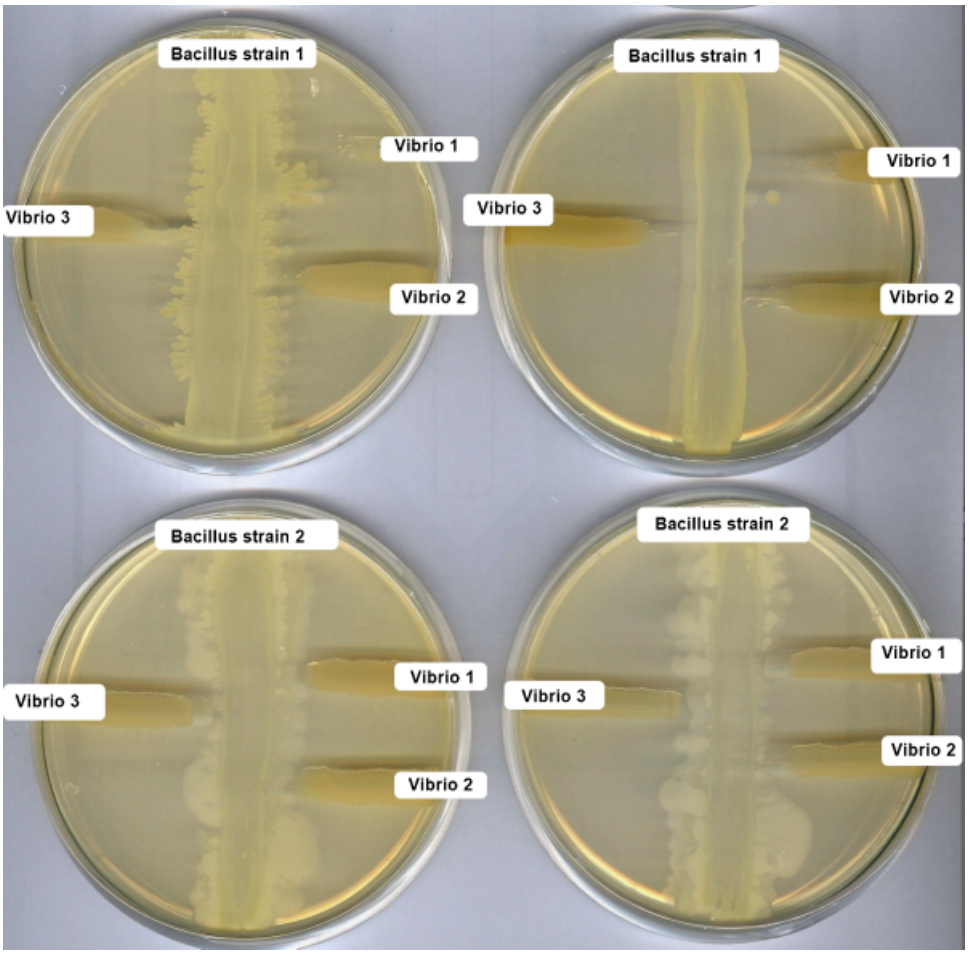


Figure 1 Evaluation of the inhibitory activity of two different *Bacillus* strains against three pathogenic *Vibrio* strains, by the cross-streak method.

Results and discussion

Several strains of *B. subtilis*, *B. licheniformis* and other species showed strong inhibitory activity against a large number of *Vibrio* strains (Fig. 1). The ability of the best-performing strains to grow in a range of salinities and denitrify nitrate to gas was also confirmed (Fig. 2).

Regarding safety for humans, it was very important to certify the absence of *B. anthrax* and *B. cereus* and toxins such as the enterotoxins and emectic toxins that are known to be produced by a range of *Bacillus* strains (From, Pukall, Schumann, Hormazábal & Granum 2005). The Oxoid bioassay confirmed the absence of the diarrhoeal toxin. We are currently investigating other tools allowing us to confirm the absence of other toxins or genes associated with the production of these toxins.

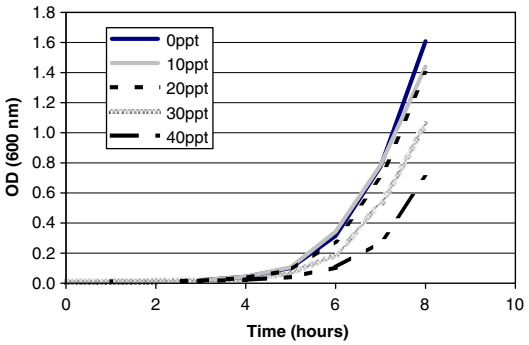


Figure 2 Growth rate of a *Bacillus* strain, estimated by measuring the absorbance at 600 nm. The *Bacillus* was grown at 0, 10, 20, 30 and 40 g L^{-1}. Two replicates per treatment.

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In vitro antagonism and laboratory-scale performance cannot completely predict an *in vivo* effect. As shown in Fig. 3, the shrimp larvae from the antibiotic and probiotic treatments performed better than those from the negative control. However, the effect of the treatment varied with each test, despite the controlled protocol, due to seasonal changes in nauplii quality, environmental conditions, the presence of particular *Vibrio* strains, etc. In two of these three tests, the tank biomass was also recorded at harvest. The tank biomass of the negative control (18.6 ± 15.6 g tank^{-1}) was significantly lower than recorded for the antibiotic (48.3 ± 22.3 g tank^{-1}) and probiotic tanks (58.3 ± 28.7 g tank^{-1}). There were no significant differences between antibiotic and probiotic treatments.

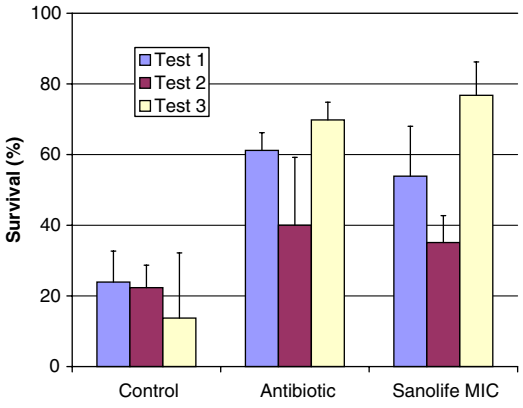


Figure 3 Survival of *Penaeus monodon* in a Thai hatchery in three separate tests carried out over a 12-month period. Treatments included negative control, prophylactic antibiotic and SANOLIFE® MIC. Four replicates per treatment.

The better performance can be partially explained by the improved water quality and notably the lower *Vibrio* load in the water (Fig. 4). In that specific test, the concentration of *Vibrio* measured in both antibiotic and probiotic treatment tanks was significantly lower than that recorded in the negative control tanks.

Similarly, the daily application of SANOLIFE® MIC to 20 MT tanks stocked with *L. vannamei* led to a marked improvement in survival compared with the negative control (Fig. 5). Unfortunately, the lack of replicates inherent to field evaluations prevented statistical analyses.

As shown in the two examples, probiotics have the potential to produce results similar to those obtained with antimicrobials and, importantly, in a cost-effective manner. Obviously, minimizing the risk of vibriosis demands a multi-disciplinary approach, including good hygiene and sanitation measures to lower the input of potential pathogens, as well as a suitable farm management (Van De Braak, Decamp & Lavens 2004). Products such as probiotics are only effective and cost-beneficial when they are properly applied together with a suitable farm management. Interestingly, this probiotic mixture also gave very promising results in mitten crab larviculture with increased survival and dry megalopa body weight.

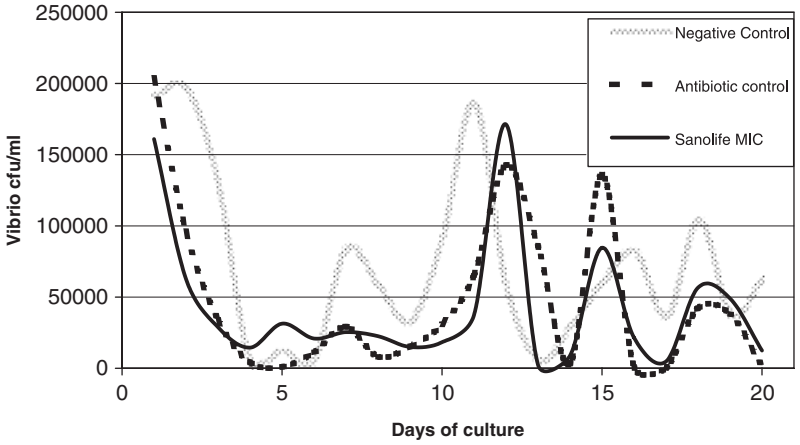


Figure 4 Daily change in abundance of *Vibrio* in water tanks, as indicated by Sanoguard® BDS-G slides, in tanks receiving antibiotics as prophylactics or daily water application of SANOLIFE® MIC, compared with a negative control. Individual points represent the average of four replicate tanks per treatment.

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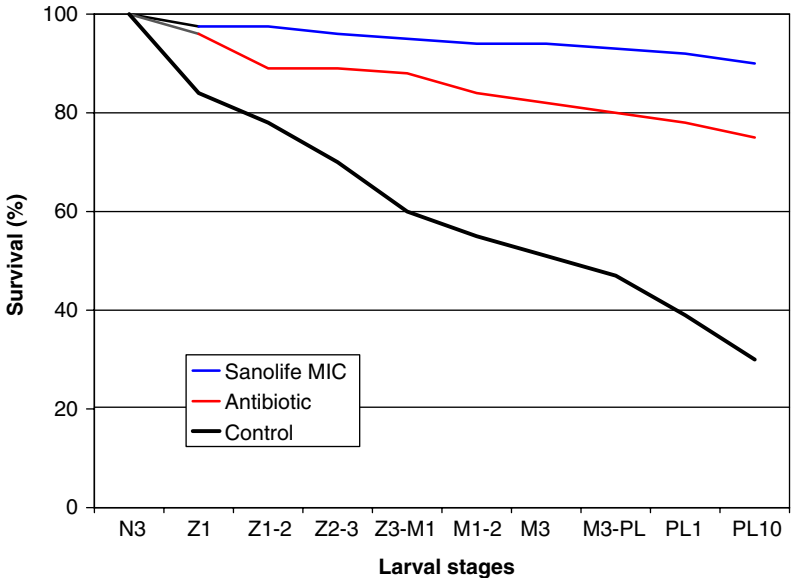


Figure 5 Survival of *Litopenaeus vannamei* larvae in a commercial Brazilian hatchery. The performance of SANOLIFE® MIC was compared with prophylactic antibiotic (in combination with formaldehyde treatment in the early postlarvae stages) and negative control (no chemicals added).

Acknowledgments

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