The Potential Use of *Bacillus* and *Lactobacillus* as Probiotics in Portunid Crab Larviculture

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FIGURE 1. *Female* Scylla paramamosain *broodstock.*

FIGURE 2. *Gram stained, rod-shaped* Bacillus licheniformis *(40 x magnification).*

Portunid crabs are commercially important crustaceans. One of the major obstacles to the growth of crab aquaculture is the low supply of hatchery-produced seed due to bacterial diseases and high mortality rates. Currently, antibiotics are used extensively in crab larviculture to improve survival, but their use poses numerous risks, including the development of antibiotic resistance in bacteria, higher levels of virulence, and accumulation of restricted chemicals in the flesh of farmed animals.

This article assesses the potential of using *Bacillus* and *Lactobacillus* as probiotics on the culture performance of crab larvae. Using probiotics is a promising way to increase survival rates and culture performance in a sustainable way. This article also discusses the negative effects of vibrio bacteria on larval crabs because vibriosis is a serious disease issue in crustacean culture worldwide.

The Use of Probiotics in Aquaculture

The focus on probiotics as an environmentally safe option for disease control is increasing and their use is now based on practical experience and research. Worldwide demand for probiotic components, supplements and feeds reached US$15.9 billion in 2008 and was around US$ 19.6 billion by 2013, with a yearly increase of 4 percent. There are now many commercial probiotic products that include single or multiple species. The need for options to the use of antibiotics is ongoing and the field of probiotics for aquaculture is now attracting considerable attention. The methods of infection by many fatal pathogens have been elaborated and experience indicates improved survival of culture animals challenged with bacterial pathogens when probiotics are used.

Probiotics can be provided as feed supplements and culture water additives. Using probiotics results in modification of bacterial communities in tank water, improving growth and survival of larval crustaceans and bivalves. Regular application of probiotics is necessary to achieve and maintain artificial dominance by the probiotic bacteria. Thus, it is feasible to alter the makeup of bacterial flora in culture through probiotic supplementation, but sustained additions are needed for the beneficial effect to occur.

Many papers have been published on the use of probiotics in aquaculture systems to control water quality, especially ammonia levels. A mixture of probiotics in solution and particle form is typically added to high-density aquaculture tanks. The mixture enhances water quality in crustacean cultures by reducing the concentration of organic matter and ammonia. Adding *Bacillus* to culture environments can enhance water quality. Indigenously isolated *B. subtilis* has been used for aquaculture. Some were isolated from shrimp ponds that had lower ammonia compared to those without probiotic application.

During passage through the digestive tract, probiotics can attach and proliferate in the gut, enhancing digestive enzyme activity and larval development. Attachment of probiotic bacteria to the intestinal surfaces of cultured animals is associated with stimulation of the immune system and inhibitory activities toward enteropathogens. Probiotics improve the digestibility of dietary energy, resulting in greater growth rates of cultured animals. Preinoculation with probiotics can prolong the dominance of the desired bacteria in the culture environment. This can lower the mortality as a result of the improved capacity of larvae to survive infections from pathogenic bacteria.

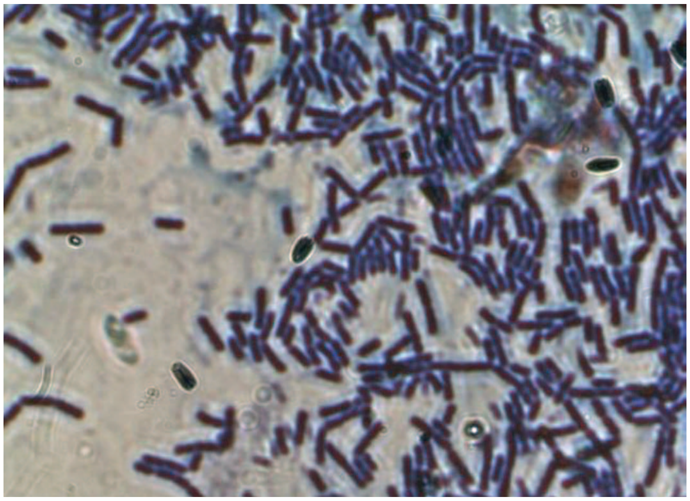

FIGURE 3. *Spore stained* Bacillus subtilis *(100 x magnification).*

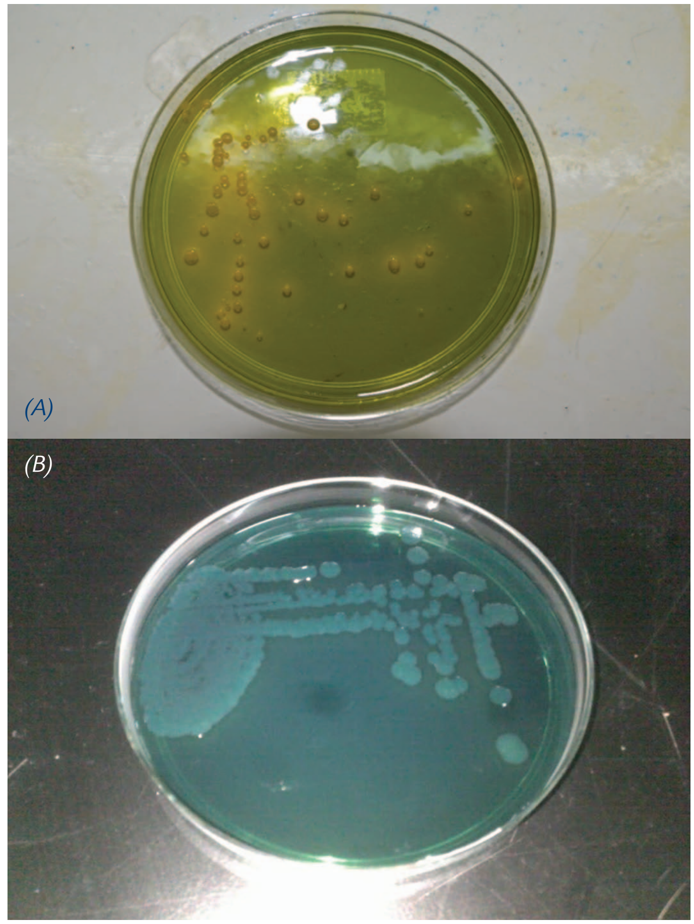

FIGURE 4. *Colony morphology of (A)* Vibrio cincinnatiensis *and (B)* Vibrio parahaemolyticus *isolated from larval culture water.*

Types of Probiotics

Various types of probiotics have been tested for crustacean culture, including Gram negative and Gram positive bacteria, bacteriophages, yeast (*Saccharomyces*) and phytoplankton. The use of *Bacillus* and *Lactobacillus* has shown tremendous potential, related to their ability to synthesize proteases and other enzymes that aid digestion in cultured animals. *Pseudomonas*, *Acinetobacter*, and *Cellulomonas* reduce the accumulation of organic matter and *Nitrosomonas* and *Nitrobacter* can control inorganic nitrogen.

Bacillus Probiotics

Bacillus is the prominent probiotic used in aquaculture to promote health and growth rates through optimization of the immune and digestive systems of cultured animals and its antibacterial activities against many pathogenic microorganisms. *Bacillus* produces endospores that are resistant to unfavorable environmental conditions. *Bacillus* inhibits pathogens in the culture water and can enhance water quality. *Bacillus* can synthesize polypeptides that are effective against a wide variety of bacteria, thus elucidating their inhibitory effects on pathogenic vibrios. The inhibitory effects of *Bacillus* are attributed to the synthesis of antibacterial proteins, antibiotics, competition for vital nutrients, and surface area. Some recommend using more than three *Bacillus* species in aquaculture feed.

Isolation and description of the antibiotic compounds in *Bacillus* will improve our understanding of their functions in bacteria. Relatively few bacteria-inhibiting toxins of *Bacillus* have been characterized, although they have tremendous potential benefits in pathogen exclusion. Zokaeifar *et al*. (2012) isolated colonies of *Bacillus* from fermented cucumbers, acetic acid, *Allium* plants, and *Brassica oleracea capitata* plants. Colonies were analyzed for their capacity to inhibit pathogenic vibrios. Two strains produced high levels of bactericidal effects toward *V. harveyi* and *V. parahaemolyticus*.

There are several commonly used *Bacillus* species for aquaculture purposes. *Bacillus subtilis* is a Gram positive, catalase positive, spore forming (the toughness of spores allows for digestive tract colonization), non-pathogenic bacteria. *Bacillus subtilis* can survive at temperatures of 11 - 52° C, 0-9 percent NaCl, and pH of 5-10. It can produce enzymes and subtilisin, which is toxic to certain bacteria.

Bacillus subtilis thrives in the gut of crustaceans and synthesizes a range of antibacterials on agar plates. *Bacillus* is non pathogenic to cultured animals, used to improve survival of cultured animals, enhances water quality, acts as an ingested probiotic treatment, and a prophylactic supplement for digestive issues. *Bacillus subtilis* also synthesizes gut proteases and other enzymes that aid in digestion.

Bacillus megaterium is used for the production of several α-amylases, β-amylases and neutral proteases, including P-amylase, P-glucanase, and megacins. It can synthesise vitamin B_{12} aerobically and anaerobically and is the major aerobic source for vitamin B_{12} for cultured animals.

Lactobacillus Probiotics

Lactobacillus is Gram-positive, non-motile and non-sporulating bacteria that produces lactic acid, has a wide range of physiology, and are catalase negative. They produce antibacterials and bacteriocins that inhibit pathogens, preventing intoxication and infections. *Lactobacillus* synthesizes protease and other enzymes that enhance the innate digestive system of cultured animals. The growth of certain *Lactobacillus* species is relatively slow because they require nutrients, a supply of carbon, nucleotides, fatty acids, amino acids and vitamins.

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Lactobacillus can contribute to a well-balanced bacterial flora in the digestive tract of culture animals, minimizing disease occurences. Although not dominant in the normal intestinal microbiota of crustaceans, some strains can colonize the gut. Artificial *Lactobacillus* populations can be maintained in the digestive tract of cultured animals through consistent supplementation. Feed polyunsaturated fatty acid levels, competition for nutrients, salinity and stress are factors that influence colonization of the gut by *Lactobacillus*. Feed is the main nutrient supply source for proliferation of certain *Lactobacillus* in the gut.

Lactobacillus is not isolated commonly from aquaculture larvae because water temperature, incubation period and environmental glucose levels can affect their density and share of the overall bacterial level. Detection of *Lactobacillus* colonies in culture water and larvae following probiotic supplementations illustrates the ability to retain these bacteria in aquaculture conditions.

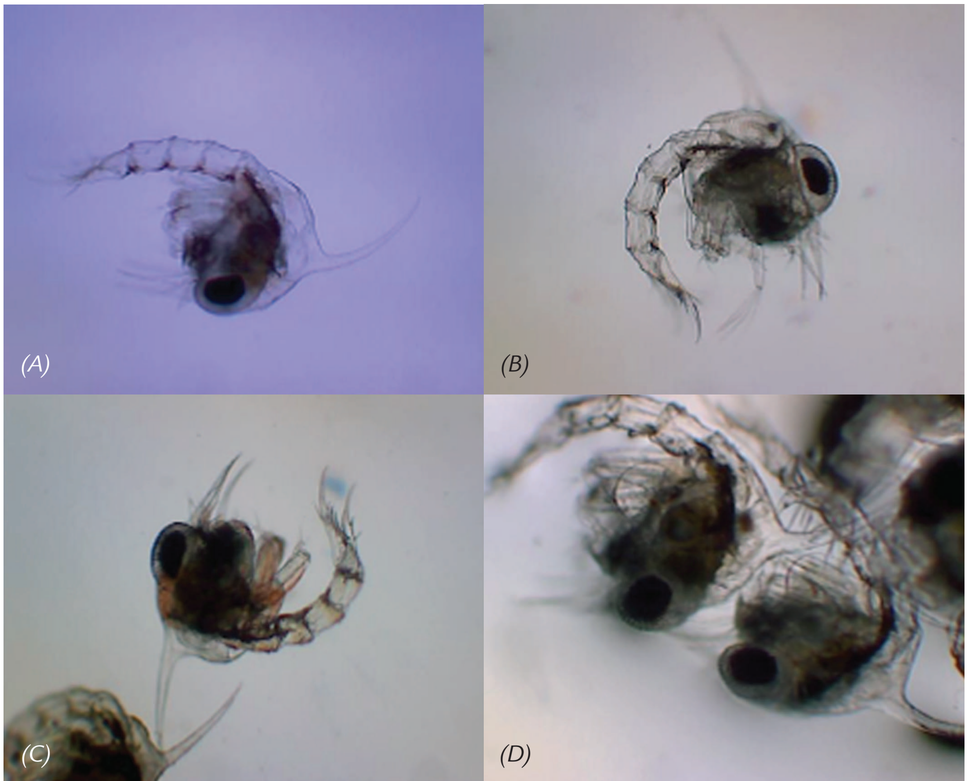

FIGURE 5. Scylla paramamosain *zoea larval stages: (A) Z1, (B) Z2, (C) Z3, (D) Z4, and (E) Z5.*

Use of Probiotics in Crab Culture

The use of probiotics to control pathogenic bacteria in crab culture has been gaining attention and various species have been tested with promising results. *Lactobacillus* was isolated from the digestive tract of blue swimmer crab *Portunus pelagicus* and used in the larval culture water (Talpur *et al*. 2012). A combination of *Lactobacillus* species (*L. plantarum*, *L. salivarius*, and *L. rhamnosus*) was inoculated into the culture water once daily at 1×10^2 cfu/mL, 5×10^2 cfu/mL, and 1×10^3 cfu/mL of culture water. *Lactobacillus* caused significantly greater survival of larvae compared to the control. The probiotics reduced the pH levels and caused a higher digestive enzyme activity compared to the control. Enzyme (protease and amylase) activities in the treated groups were greater than in the controls, indicating the effectiveness of *L. plantarum* in improving digestive enzyme activity.

Probiotics can provide supplemental digestive enzymes, improve larval development, and FCR, and reduce gut diseases. Throughout larval developmental, probiotic bacteria can grow in the digestive tract of larvae, while utilizing carbohydrates and synthesizing amylase, protease, and lipase. A mixture of *Lactobacillus* species gives additional protection during unfavorable conditions and invasion by pathogenic bacteria in *P. pelagicus* larvae (Talpur *et al*. 2012). Improved defenses of larvae when several species of probiotics were used together may be the result of each species providing a specific advantage, resulting in a wider range of beneficial effects.

The greatest enzyme activity in *P. pelagicus* occurred with mixtures of three *Lactobacillus* species at 1×10^3 cfu/ml followed by 5×10^2 cfu/ml (Talpur *et al*. 2012). *Portunus pelagicus* larvae were offered live feed, suggesting the zooplankton prey had ingested probiotics from the treated culture water, improving immunity and digestion in crab larvae. Digestion in larval marine crabs was activated during the early growth phase, during which *Lactobacillus* secreted a wide variety of enzymes. Activities of protease and amylase were significantly greater in crab larvae in tanks receiving the mixture of *Lactobacillus* compared to the control (Talpur *et al*. 2012). It was likely that probiotics influenced digestion by enhancing beneficial bacterial populations, enzyme activity, intestinal bacterial balance, digestibility, and nutrient absorption, resulting in improved survival rates from the enhanced capability of larvae to cope with pathogens.

Pathogenic Effects of *Vibrios*

Early *Scylla* larvae are feeble, have an under-developed immune system and are vulnerable to pathogenic bacteria. *Vibrio* is one of the most important pathogens of cultured aquatic crustaceans and have been implicated as the principal disease in crustacean larviculture facilities. *Vibrio parahaemolyticus* affects juvenile and adult crabs. *Vibrio harveyi* and *V. parahaemolyticus* are highly pathogenic to

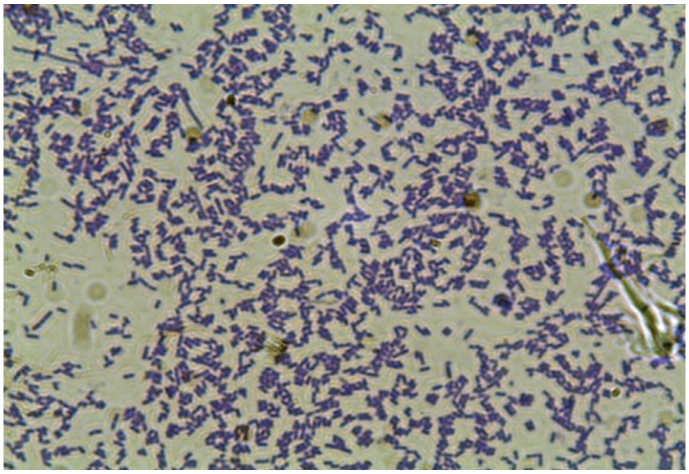

FIGURE 6. *Laboratory-cultured, Gram-stained* Lactobacillus plantarum *probiotic (100 x magnification).*

Scylla and *P. pelagicus* (Parenrengi *et al*. 1993). Luminescent *V. harveyi* (CLM3) are pathogenic to zoea (Z1) of *S. serrata*, inducing mortality of 63-64 percent at an initial dose of 10^2 and 10^3 cfu/mL, compared to 40 percent mortality in the control (Lavilla-Pitogo *et al*. 2002). Infected larvae had plagues of bacteria on the mouth and feeding apparatus, implying an oral route of entry for the pathogen.

The sources of luminescent *V. harveyi* were determined from different hatchery components (Lavilla-Pitogo *et al*. 1992). The bacterial flora of the midgut of broodstock contained 16-17 percent luminescent *Vibrio* populations and broodstock were significant sources of luminescent *Vibrio*. Broodstock crabs release large amounts of fecal material during spawning, thus facilitating bacterial colonization of newly spawned eggs. A second pathway for pathogenic bacteria to enter the culture environment of *Scylla* zoea is through live planktonic food as bacteria enter the digestive tract of larvae orally.

Furthermore, the bacterial ecology of *Scylla* larval cultures are unstable between and within culture cycles, including changes in sucrose negative and positive *Vibrio* ratios. Constant high numbers of green (sucrose negative) *Vibrio* colonies on TCBS agar is regarded as a sign that high mortality is imminent.

Lactobacillus used in aquaculture synthesizes proteases and amylases. In *P. pelagicus*, increased digestive enzyme activity of larvae in tanks receiving probiotics results in reductions of *Vibrio* in the gut of larvae and culture water, providing favorable conditions that contribute to improved larval survival and enzyme activities. Supplementation of *Lactobacillus* has an influence on the swimming behaviour of larvae, especially during the first days after hatching. *Thalassobacter utilis* (strain PM4) has been used to control *V. harveyi* and improve survival of larval swimming crab *Portunus trituberculatus*.

Vibrio alginolyticus is an environmental probiotic that protects against vibriosis in *Scylla*. The strain *V. alginolyticus* LLB2 has been isolated and tested against a control in larval *S. serrata*, both challenged with *V. harveyii* (LLD1, pathogenic strain), with the treatment group giving a higher survival rate. The use of *Bacillus* and *Lactobacillus* as probiotics is an ideal alternative to antibiotics in crab hatcheries to provide a consistent, commercially feasible production of seedlings.

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Notes

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