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Biological control of streptococcal infection in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) using filter-feeding bivalve mussel *Pilsbryoconcha exilis* (Lea, 1838)

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Summary

Since bivalve mussels are able to graze heavily on bacteria, in this paper it is hypothesized that when mussels are cultured with fish, the filtering efficiency of the mussels will keep the bacterial population below a certain threshold and thus assist in reducing the risk of bacterial disease outbreaks. The ability of the filter-feeding bivalve mussel *Pilsbryoconcha exilis* to control *Streptococcus agalactiae* was tested in a laboratory-scale tilapia culture system. Juvenile Nile tilapia (*Oreochromis niloticus*), the bivalve mussel as well as the bacteria were cultured at different combinations using four treatments: treatment-1: mussel and bacteria but no fish, treatment-2: tilapia and mussel but no bacteria, treatment-3: tilapia and bacteria but no mussel, and treatment-4: tilapia, mussels, and bacteria. All treatments were run in three replicates; stocking rates were 10 tilapia juveniles; five mussels; and about 3.5×10^5 colony forming units (CFU) ml^{-1} of bacteria in 50-L aquaria with 40-L volume. The mussel reduced the bacterial population by 83.6–87.1% in a 3-week period whereas in the absence of the mussel, the bacterial counts increased by 31.5%. Oresence of the mussel also resulted in significantly higher growth and lower mortality of tilapia juveniles than when the mussel was absent. The results of this experiment suggest that the freshwater mussel *P. exilis* could control the population of *S. agalactiae* in a laboratory-scale tilapia culture system. Future studies should focus on the dynamic interactions among fish, mussels, and bacteria as well as on how input such as feed and other organic materials affect these interactions.

Introduction

The Nile tilapia, *Oreochromis niloticus*, contributes about 83% of the total global tilapia production (Gupta and Acosta, 2004; Bostock et al., 2010). In view of the increasing commercialization and continuing growth of the tilapia industry, it has been described as the most important aquaculture species of the 21st century (FAO, 2012). Intensive tilapia production systems often experience frequent occurrence of disease due to water quality problems and high stocking densities. Early reports suggest that since the 1990s, bacterial diseases, particularly streptococcal infections (streptococcosis) have appeared as major problems in tilapia farming worldwide and resulted in an estimated annual loss of 150 million USD (Shoemaker and Klesius, 1997; Shoemaker et al., 2000). Massive mortalities of tilapia associated with streptococcosis have also been reported in recent years (Neely et al., 2002; Najiah et al., 2012). Streptococcal infections in fish are an additional concern due to reports of zoonotic infections caused by *Streptococcus* (Weinstein et al., 1997; Bowser et al., 1998; Neely et al., 2002).

Various options are available to control bacterial diseases. Good general hygiene and vaccinations are likely to reduce the risks of a disease outbreak (Klesius et al., 2008). However, performance of vaccines has been reported to vary considerably, depending on many factors (Amal and Zamri-Saad, 2011); there are also reports of vaccine failure (Bachrach et al., 2001). Although antibiotics such as oxytetracycline (incorporated into feed) were found to be effective in controlling streptococcosis in blue tilapia (Darwish and Griffin, 2002), others have also been tested but with unsatisfactory results (Najiah et al., 2012). Another significant problem with antibiotics is that the bacteria soon develop resistance (Darwish and Hobbs, 2005; Defoirdt et al., 2011; Najiah et al., 2012). Furthermore, there are growing concerns over the detrimental consequences of using antibiotics in aquaculture (Holmström et al., 2003; Serrano, 2005; Defoirdt et al., 2011).

One potential approach is that of biological control using organisms to reduce the bacterial load by grazing on them so that the bacterial population cannot flourish to a level capable of causing disease. Bivalves are filter feeders and are well known for grazing heavily on phytoplankton, bacteria, and resuspended particulate matter with attached microflora (Cotner et al., 1995). Bivalves, particularly mussels, have been reported to graze heavily on bacteria that subsequently contribute significantly to the nutrition for mussel (Birkbeck and McHenery, 1982; Cotner et al., 1995; Silverman et al., 1995; Kreeger and Newell, 1996). In this experiment, we tested the ability of a freshwater mussel *Pilsbryoconcha exilis* to prevent streptococcosis in Nile tilapia *Oreochromis niloticus* with the hypothesis that when the mussel is cultured together with fish, the filtering efficiency of the mussel will reduce the bacterial population threshold and thus assist in reducing the risk of disease outbreaks.

Materials and methods

Juvenile Nile tilapia (*Oreochromis niloticus*; mean weight 1.80 ± 0.02 g; n = 120) were produced in the hatchery and the freshwater mussel *Pilsbryoconcha exilis* (mean length, 12–16 cm) was collected from Lake Kenyir, Terengganu. The *Streptococcus agalactiae* used to infect the experimental fish was isolated from an infected cultured tilapia from Lake Kenyir. The bacteria species was confirmed by using the BBL Crystal Identification Systems Gram-Positive ID Kit (Becton, Dickinson and Company). Aliquots of the isolated *S. agalactiae* were grown by swabs directly onto Granada agar (Hardy Diagnostic, Santa Maria, California) which was incubated at 37°C (Overman et al., 2002). Plate counts were done according to the drop plate method (Herigstad et al., 2001) on dilutions of the stock culture to determine the concentration of *S. agalactiae*. Aliquots of bacteria samples were taken and serially diluted 10-fold. Ten microliters (μl) of these dilutions were added (five drops per dilution) to Granada agar plates using the drop plate method, incubated at 37°C for 3 days, and colonies were counted after incubation.

The experiment included four treatments. Treatment-1 (T-1) contained mussel and bacteria but no fish; Treatment-2 (T-2) contained tilapia and mussel but no bacteria; Treatment-3 (T-3) contained tilapia and bacteria but no mussel; and Treatment-4 (T-4) contained tilapia, mussels, and bacteria. All treatments were run in three replicates; stocking rate 10 tilapia juveniles; five mussels; and about 3.5×10^5 colony forming units (CFU) ml^{-1} of bacteria in 50-L aquaria with 40-L volume.

Fish were fed a commercial pelleted diet for 21 days at a rate of 4% of the body weight daily and in two rations. The mussel was fed a pure culture of *Chorella* sp. at an approximate rate of 1.5×10^5 cells ml^{-1} per day. An aliquot of *S. agalactiae* was inoculated into 400 ml of Tryptic Soy Broth (TSB) and incubated in a water bath at 30°C until the bacteria reached the mid-log phase of growth (concentration approximately 10^9 CFU ml^{-1}). The bacteria were harvested by high speed centrifugation at 10 000 rpm for 10 min and the supernatant was discarded. The isolate was re-suspended in 50 ml of the same tap water in which the fish and/or mussels were being held; concentration of bacteria in this 50 ml stock was estimated as approx. 4.0×10^9 CFU ml^{-1}. This bacterial stock was inoculated into the experimental tanks at a rate of 3.5 ml stock per tank (40 L water) so that the bacterial concentration in the tank water was approx. 3.5×10^5 cells ml^{-1}.

Fish in each tank were observed daily for any visible symptoms of bacterial infection such as erratic swimming, sluggishness, hemorrhagic eyes, abdominal distension or mortality. The number of dead fish was recorded daily to calculate the mortality rate expressed as percentage of the number initially stocked. Dead fish with visible symptom were removed from the tank and the bacteria isolated from the infected area onto Granada agar and later checked with a BBL kit to confirm the cause of death as being by *Streptococcus*. Tilapia weight gain was computed as the difference between initial and final weight. Specific growth rate (SGR) of the surviving fish was calculated as:

$$SGR = [\ln(W_2) - \ln(W_1)] \div (t_2 - t_1) \times 100$$

where, W_1 and W_2 are the mean weight of fish at stocking and at termination respectively; $t_2 - t_1$ is the length of the experiment (in days).

All mussels were checked for mortality. Indicators used to differentiate between live and dead mussels are the condition of the valves (open or closed) and the body color. A live mussel will stay intact, without opening the valve, in contrast to a dead mussel that loses the ability to control the muscle to keep the shell closed. A dead mussel will also turn darker (black) in color than that of a live mussel.

Triplicate water samples were taken from each tank for bacterial counts every 2 days, from day-4 to day-20 (the experiment was terminated on day 21). Samples were processed for bacterial counts as previously described. Only concentrations of the inoculated bacteria, *S. agalactiae*, were determined in the water samples, assuming that the other bacteria contributed only a minor portion of the total bacterial counts and that the concentrations of other bacteria did not differ significantly among experimental tanks. Initial and final bacterial counts were compared for each treatment, and the decrease in bacterial concentration was compared among treatments. Major water quality parameters such as temperature, salinity, dissolved oxygen and pH were measured in each tank every 2 days.

Variations in tilapia mortality, weight gain, SGR, final bacterial count, and concentration decrease of bacteria among experimental treatments were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* multiple comparison tests for significant parameters. Percentage data (cumulative mortality and concentration decrease of bacteria) were arcsin (square root) transformed before analysis. Initial and final bacterial counts were log-transformed and compared using a paired *t*-test. All statistical analyses were performed on SPSS version 20.0.

Results

Temperature (°C), dissolved oxygen (mg L^{-1}) and pH ranged from 28.26 ± 0.01 to 28.28 ± 0.01, from 5.38 ± 0.08 to 5.43 ± 0.09, and from 7.12 ± 0.22 to 7.46 ± 0.012, respectively, and did not differ significantly among treatments. In T-1 and T-4 where the mussels and bacteria were cultured together, the bacterial population decreased in concentration consistently throughout the experimental period (Fig. 1). In contrast, in T-3, which had no mussels, the bacterial population increased slowly in concentration steadily throughout the experiment. Initial and final bacterial counts differed significantly in all three treatments (Fig. 2). In T-1 and T-4, final bacterial counts were significantly ($P < 0.001$) lower than initial counts while in T-3, final counts were significantly ($P < 0.001$) higher than the initial bacterial counts. On average, bacterial population was reduced by 87.1% in treatment-1 and 83.6% in treatment-4, and increased by 31.5% in T-3.

The lowest cumulative mortality (16.7%) and highest specific growth rate (1.13 ± 0.11) of tilapia were recorded in T-2 (Figs 3 and 4) where no bacteria were added. In

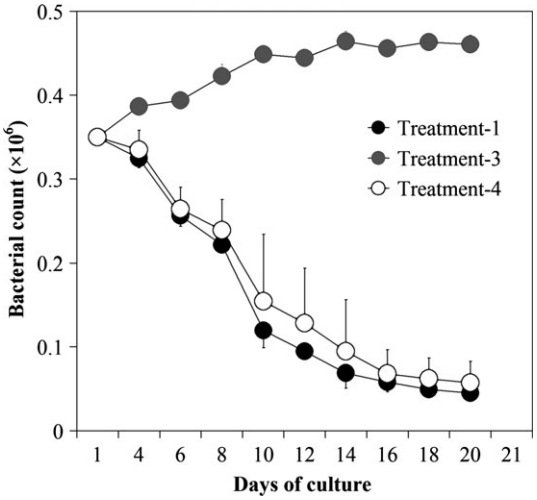

Fig. 1. Changes in bacterial counts in culture water with time (days of culture); each data point represents the mean (±SD) value for three replicates. Treatment-1: mussel + bacteria, and no fish; Treatment-3: fish + bacteria, and no mussels; Treatment-4: fish + mussels + bacteria. No data for Treatment-2, as the experimental bacteria in Treatment-2 was not inoculated and not all bacteria in the water were counted (see 'Materials and methods')

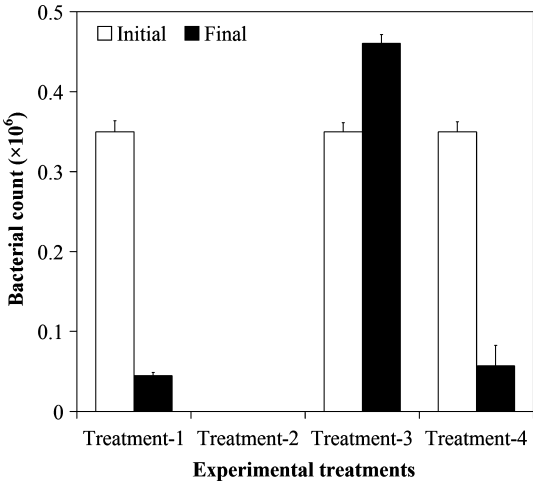

Fig. 2. Initial and final (at the end of the experiment on day-21) bacterial count in culture water in different treatments; each data point represents the mean (±SD) value for three replicates. Treatment-1: mussels + bacteria, and no fish; Treatment-2: fish + mussels, and no bacteria; Treatment-3: fish + bacteria, and no mussels; Treatment-4: fish + mussels + bacteria

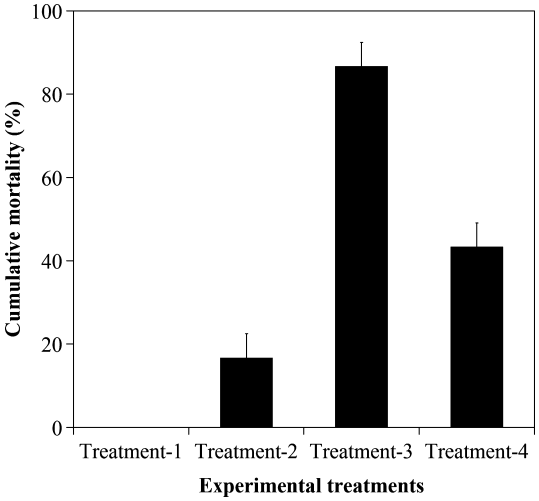

Fig. 3. Cumulative mortality rates (%) of tilapia juveniles cultured for 21 days with different combinations of tilapia, mussels, and bacteria; each data point = mean (±SD) value for three replicates with initially 10 fish each. Treatment-1: mussels + bacteria, and no fish; Treatment-2: fish + mussels, and no bacteria; Treatment-3: fish + bacteria, and no mussels; Treatment-4: fish + mussels + bacteria

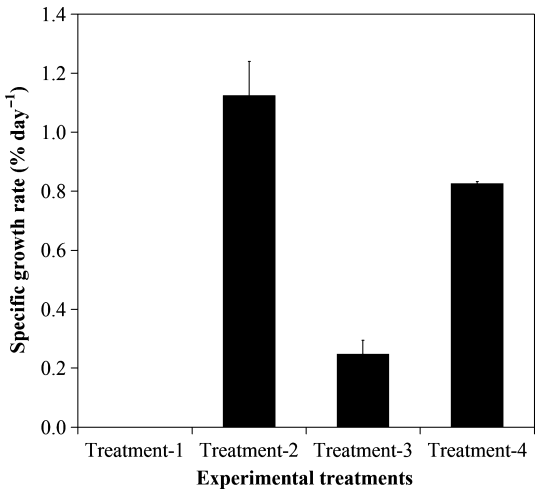

Fig. 4. Specific growth rate (% day^{-1}) of tilapia juveniles cultured for 21 days with different combinations of tilapia, mussels and bacteria; each data point = mean (±SD) value for three replicates (initially 10 fish each). Treatment-1: mussels + bacteria, and no fish; Treatment-2: fish + mussels, and no bacteria; Treatment-3: fish + bacteria, and no mussels; Treatment-4: fish + mussels + bacteria

contrast, highest cumulative mortality (86.7%) and lowest specific growth (0.25 ± 0.05) were recorded in T-3 (Figs 3 and 4) in which tilapia was infected with bacteria. Bacteriological observations confirmed that the cause of death of tilapia was *Streptococcus* bacteria. Addition of mussel with tilapia and bacteria in T-4 resulted in significantly ($P < 0.001$) lower mortality (43.3%) and higher specific growth (0.83 ± 0.01) of tilapia than in T-3 (Figs 3 and 4).

Clinical signs started to appear beginning on day-3 after the experiment began; signs included hemorrhages at the fin base, eyes and operculum, loss of appetite as evident from reduced feeding, a distended abdomen and erratic swimming. Once the fish started to exhibit clinical signs, an estimated reduction in feed uptake of 50% was observed in T-3 and 25% in T-4. Similarly, the dead fish in T-3 had abdomens about 80% larger than normal; the abdomens in T-4 fish were approx. 164% larger than normal. No fish in T-2 showed clinical signs of disease or a reduction in feed intake. All individual fish died that showed clinical signs, whereby

death began on day-5 of the trial and continued until day-14 when clinical signs became less evident. No mussels died in any of the experimental tanks.

Discussion

Streptococcus spp. have been reported as a major problem in tilapia farming, causing mass mortality and significant economic losses in tilapia aquaculture (Shoemaker and Klesius, 1997; Shoemaker et al., 2000; Najiah et al., 2012). The disease has also caused significant problems in other aquaculture industries, particularly salmonids (Romalde and Toranzo, 2002). *Streptococcus iniae* and *Streptococcus agalactiae* are the two major species affecting tilapia aquaculture worldwide (Najiah et al., 2012). Streptococcal infection produces a range of clinical signs in fish including hemorrhaging, loss of appetite, spinal displacement, corneal opacity, exophthalmia (pop-eyes), distended abdomen, darkening of the skin, and erratic swimming. One common streptococcosis appearance is that of meningoencephalitis, where large numbers of bacteria accumulate in the central nervous system of the infected fish, resulting in quickly occurring mortality (Eldar et al., 1994, 1995).

Vaccines are reported to provide good levels of protection but only if administered by intraperitoneal injection (Håstein et al., 2005; Toranzo et al., 2005), which is time-consuming and expensive as well as stressful for the fish. Use of antibiotics, on the other hand, is under tight criticism because of a range of direct and indirect detrimental consequences (Teuber, 2001; Burridge et al., 2010; Defoirdt et al., 2011; Romero et al., 2012; Cabello et al., 2013). In addition, both vaccines and antibiotics add significantly to the management costs.

The specialized filtration mechanisms of the gills allow mussels to capture particles <1 μm in size, such as bacteria (Silverman et al., 1996, 1997), while their enzyme lysozyme (Mchenery et al., 1983) can affect lysozyme-sensitive bacteria rapidly (Birkbeck and McHenery, 1982). Antibacterial activities of plasma samples from mussels have been reported (Anderson and Beaven, 2001) and antimicrobial peptides have also been isolated from mussels (Charlet et al., 1996). Bacteria provide a significant source of nutrients for mussels (Kreeger and Newell, 1996, 2001). Mussels not only graze on bacteria but also assimilate them at a much greater rate than when they assimilate the algae (Silverman et al., 1996), suggesting that bacteria are a significant source of carbon for mussels (Nichols and Garling, 2000).

Our study showed that the presence of mussels could reduce the growth of pathogenic *Streptococcus* in a laboratory-scale tilapia culture system. In a simulated polyculture system involving green mussels, brown mussels, oysters, and shrimp, Tendencia (2007) showed that the growth of luminous bacteria (*Vibrio harveyi*) decreased from 10^4 CFU ml^{-1} to below 10^1 CFU ml^{-1} after different durations depending on the density of the bivalves, and helped control diseases caused by luminous bacteria. Jones and Preston (1999) reported that bacterial numbers in the effluent from a shrimp pond were reduced by 58% in ponds stocked with oysters, whereas no significant effect on the bacterial concentration in control ponds was observed. Antibacterial activities in mussels and other bivalves have also been reported in other studies (Anderson and Beaven, 2001). Overall, mussels cleared over 85% of the bacterial population in 3 weeks. In our study, we speculate that the mussels would have completely cleared the bacteria from the culture water if the trial had been continued for another week. However, this may not be achievable under commercial culture conditions.

In conclusion, the results of this short-term experiment demonstrated that the freshwater mussel *Pilsbryoconcha exilis* could significantly reduce the concentration of *Streptococcus agalactiae* in a laboratory-scale tilapia culture system. Detailed future studies are necessary to understand the dynamic interactions among the fish, mussel and bacteria as well as the effects of aquaculture input such as feeds and other organic materials. Field trials are necessary to explore the utility of mussel inclusion to control streptococcosis in actual aquaculture situations. Potentially, this biological control method could be economically beneficial and at the same time environmentally safe, and would help promote the overall sustainability of the aquaculture industry.

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