Research Article

Probiotic effects on cobia *Rachycentron canadum* larvae reared in a recirculating aquaculture system

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ABSTRACT. Cobia (*Rachycentron canadum*) is a marine finfish with good potential for mariculture. This study analyzes the effects of probiotic *Bacillus* spp. on the performance of cobia larvae reared in a recirculating aquaculture system (RAS). Larvae were stocked into two independent RAS for 26 days after hatching. One of the systems (Probiotic treatment) received the addition of a commercial probiotic consisting of *B. subtilis*, *B. licheniformis* and *B. pumilus* directly into the water and by live feed. Survival, final weight and water quality were not affected by probiotics. Results showed larvae of the probiotic treatment demonstrated a greater resistance to salinity stress. Immunohistochemical analysis showed a higher expression of CD4 in probiotic treatment. These results suggest that *Bacillus* spp. probiotics used in RAS have a potential stimulating impact on immune system differentiation and increases salinity stress resistance of cobia larvae.

Keywords: *Rachycentron canadum*, cobia, *Bacillus*, aquaculture, immunostimulation, immunohistochemistry, mariculture.

Efectos probióticos sobre las larvas de cobia *Rachycentron canadum* criadas en un sistema de recirculación de agua

RESUMEN. Cobia (*Rachycentron canadum*) es una especie de pez marino con buen potencial para la maricultura. Este estudio analiza los efectos del probiótico *Bacillus* spp. sobre el desempeño de las larvas de cobia criadas en un sistema de recirculación de agua (RAS). Las larvas fueron sembradas en dos RAS independientes durante 26 días después de la eclosión. Uno de los sistemas (tratamiento experimental) recibió la adición de un probiótico comercial que consiste en *B. subtilis*, *B. licheniformis* y *B. pumilus* directamente en el agua y por alimento vivo. La sobrevivencia, peso final y calidad del agua no se vieron afectados por los probióticos. Los resultados mostraron que las larvas del tratamiento probiótico presentaron una mayor resistencia al estrés por estrés salino. El análisis inmunohistoquímico mostró una mayor expresión de CD4 en el tratamiento probiótico. Estos resultados sugieren que estos probióticos utilizados en las larvas de cobia en RAS tienen un potencial impacto estimulante sobre la diferenciación del sistema inmunológico y aumenta la resistencia al estrés de las larvas de cobia.

Palabras clave: *Rachycentron canadum*, cobia, *Bacillus*, acuicultura, inmunoestimulación, inmunohisto-químico, maricultura.

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INTRODUCTION

Cobia (*Rachycentron canadum*) is a marine finfish that has an emerging global potential for mariculture due to its extraordinary growth rate (Sampaio *et al.,* 2011) and overall aquaculture performance (Holt *et al.,* 2007).

Aquaculture environments can be stressful, triggering high mortality, especially during larval rearing (Avella *et al.,* 2010). Typical intensive larviculture production protocols can cause disease by opportunistic pathogens (Hansen & Olafsen, 1999) and in order to avoid these potential diseases and antimicrobial drugs, the use of probiotics in aquaculture is suggested (Gatesoupe, 1999; Velmurugan & Rajagopal, 2009). *Bacillus* spp. probiotics are commonly used in animals and the beneficial roles of *Bacillus* spore applications

in the aquaculture field are well established (Queiroz & Boyd, 1998).

Probiotics can be defined as live microbial feed supplements that can improve host intestinal microbial balance of the host and in turn its health (Gatesoupe, 1999). It has also other beneficial effects, as improvement of water quality (Wang *et al.,* 2008). In aquaculture, probiotics can be inoculated into the rearing water or incorporated into the dry or live feed (Taoka *et al.,* 2006).

Several positive effects of probiotics in fish culture have been demonstrated, such as higher survival (Velmurugan & Rajagopal, 2009), faster growth (Aly *et al.,* 2008), improved stress tolerance (Rollo *et al.,* 2006), immune system enhancement (Picchietti *et al.,* 2009; Cha *et al.,* 2013), and general welfare (Silvi *et al.*, 2008). These multiple effects of probiotics confirm their successful use in fish husbandry areas including nutrition, environmental control and immunity (Lazado & Caipang, 2014).

The effects of probiotics on immune response include the enhancement of non-specific immune responses (Taoka *et al.,* 2006) and increase of T-cells (Picchietti *et al.,* 2009). Some immunohistochemical markers can be used to study leukocyte populations, like the T-cell co-receptors CD3 and CD4 that are essential for cell-mediated immunity (Laing *et al.,* 2006). The CD4 co-receptor occurs in T-helper cells, which play a central role in the immune system (Romano, 2010). Immunohistochemistry is an important technique that can be used to evaluate fish immunity although most studies on probiotics in fish culture focus on pathogen challenge (Picchietti *et al.,* 2007).

Recirculating systems can be used to maximize biosecurity and seasonal environmental influences (Lyndon, 1999) in marine larviculture. However, few studies have evaluated the use of probiotics in these systems (Chen & Chen, 2001; Taoka *et al.,* 2006). Thus, this study aims to examine the effects of a probiotic composed of three *Bacillus* strains on survival, growth, immunostimulation, and resistance to a salinity stress challenge in cobia larvae reared in a RAS.

MATERIALS AND METHODS

The experiment was conducted at Virginia Tech's Virginia Seafood Agricultural Research and Extension Center (VSAREC) (Hampton, VA, USA). Two independent RAS were used in this experiment, each comprised of three tanks (300 L each) (Holt *et al.,* 2007). The probiotic treatment and the control were randomly assigned to one recirculation system each (n = 3); because of the nature of the study, tanks could not be randomized among systems.

Fertilized cobia eggs were transported from Trout Lodge Marine Farms (Vero Beach, FL, USA) to the VSAREC. Eggs were incubated until hatching and newly hatched larvae were stocked into the experimental tanks at a density of 15 larvae per liter.

Larvae were produced according to standard larviculture production protocols (McLean *et al.*, 2009). They were maintained in a green water system, by algal paste additions (Instant Algae Nanno 3600, Reed Mariculture, Campbell, CA, USA) in a concentration of 10^6 cells mL^{-1}. L-type rotifers were enriched for 6 h with Protein Selco Plus (INVE, Salt Lake City, UT, USA) and were fed from 2 through 9 dph. Newly hatched AF *Artemia* (INVE, Salt Lake City, UT, USA) were fed from 7 through 10 dph and enriched GSL *Artemia* (INVE, Salt Lake City, UT, USA) were fed from 10 through 23 dph. *Artemia* were enriched for 24 h with DC DHA Selco (INVE, Salt Lake City, UT, USA). Co-feeding of larvae with Otohime Marine Larvae Weaning Diets (Reed Mariculture, Campbell, CA, USA) began on 15 dph, weaning protocols began on 21 dph, and all fish were fully weaned and *Artemia* discontinued by 23 dph.

Flow rate in the RAS was maintained at 60 L h^{-1} from stocking through 9 dph. On 10 dph, flow increased to 90 L h^{-1}; on 12 dph, flow was increased to 120 L h^{-1}; on 13 dph, flow increased to 180 L h^{-1}; on 16 dph, flow was increased to 240 L h^{-1} and on 18 dph flow increased to 300 L h^{-1} and remained there for the rest of the study.

The photoperiod was maintained at 24 h light using fluorescent light banks. Initial system salinity was 35 g L^{-1}, which was reduced 1 g L^{-1} per day until a final salinity of 22 g L^{-1} was achieved at 13 dph.

At the end of the experiment (26 days post hatch), 150 fingerlings were randomly sampled from each tank to determine final individual weight. All remaining larvae from the six experimental tanks were counted to determine survival.

A commercial *Bacillus* mixture provided by INVE Technologies (Belgium) was used. It is composed of *B. subtilis*, *B. pumilus* and *B. licheniformis* ($1x10^{10}$ CFU g^{-1}). Each Probiotic treatment tank received a daily probiotic dose of 5 g m^{-3} directly into the water ($5x10^4$ CFU mL^{-1}), equally divided into four applications. Live feeds for this treatment were also enriched with the probiotic, adding 0.5 g L^{-1} of enrichment medium 6 h prior to harvesting, according to the manufacturers specifications.

Temperature, salinity, pH, dissolved oxygen, alkalinity, total ammonia-nitrogen (TAN), nitrite (NO_2), and

nitrate (NO$_3$) were monitored daily. Water temperature ranged from 27.9-28.5°C, dissolved oxygen concentration was maintained above 6.1 mg L^{-1} and pH was 7.9 ± 0.1. Alkalinity ranged from 142-186 mg L^{-1}.

Water samples for microbial analysis were taken daily and determined by plating 1 mL water sample from each tank. The culture medium Trypticase™ Soy Agar 2% NaCl was used for total spore-forming bacteria abundance after sample pasteurization in order to eliminate non-spore-forming vegetative cells (Miskin *et al.*, 1998), assuming that most of the spore-forming cells were *Bacillus*. For total *Vibrio*, the samples were placed on Thiosulfate Citrate Bile Salt Sucrose agar. Abundance was expressed in colony-forming units per mL (CFU m L^{-1}) according to Banwart (1989).

After 26 days, a stress challenge was carried out according to Dhert *et al.* (1992). Ten fish per tank were transferred to 2 L-beakers at 60 ppt salinity and survival was monitored at 5-min intervals until mortality reached 100%. The sensitivity to the stress of salinity was expressed by the Stress Sensitivity Index (SSI), which was calculated as the sum of cumulative mortality observed over the test period (Dhert *et al.,* 1992). The higher numeric value of the index indicates greater mortality of the larvae, which denotes that they were less able to tolerate the stress of the salinity shock.

At the completion of the study ten fish per tank were sampled for immuno histochemical analysis of the thymus. They were fixed in 10% buffered formalin, embedded in paraffin and sections stained with haematoxylin and eosin. In addition histological sections were also stained using immuno histochemical procedures, according to a modified avidin-biotin-peroxidase complex technique (Hsu *et al.*, 1981; Prophet *et al.*, 1992). This involved tissue slides being deparaffinised, rinsed with xylol and then rehydrated with alcohols of different grades (absolute ethanol, 90, 80, 70, 50%). The endogenous peroxidase activity was blocked by incubating the slides for 20 min in 0.3% H$_2$O$_2$ in a 5% methanol solution. After washing the slides in water and PBS/0.05%-Tween 20 solution, they were incubated in normal 1/100 serum (Vectastain Universal Elite, BC Kit, Vector) in a 10% PBS bovine serum albumin (BSA) solution at normal temperature for 30 min in a humid chamber. After incubation, the primary anti- CD3 a CD4 antibody (Dako, Argentina) was added at a dilution of 1:200 in PBS-BSA 10%, and the slides were incubated overnight at 41°C in humid chamber. This was followed by rinsing and incubation for 7 min in a 50 mL 30.3-diaminobenzidine (DAB, Sigma-Aldrich) solution containing 1% PBS-BSA in 50 mL H2O2. Counterstaining was then performed with haematoxylin. The CD3 and CD4 receptors were

submitted to quantitative analysis of phenotype expression percentage per square millimeter of tissue (Romano *et al.*, 1996) using the software Bioscan OPTIMAS® 6.1 (Weibel, 1979).

All data was compared using a Student's T test with Statistica® 7.0 (StatSoft, Inc., Tulsa, OK, USA). Differences were considered significant at $P < 0.05$.

RESULTS

Throughout the experimental period all water quality parameters were maintained within suitable ranges for cobia. There were no significant differences ($P > 0.05$) in survival, final weight, water quality and total *Vibrio* counts between treatments. Nevertheless, total count of spore-forming bacteria in the probiotic treatment differed statistically, with a higher concentration in the probiotic treatment ($P < 0.05$) (Table 1).

In the stress challenge, the probiotic treatment had lower Stress Sensitivity Index than the control ($P < 0.01$) (Fig. 1).

The expression of CD3 in thymus tissue showed no statistical differences between groups. However, CD4 expression was significantly higher ($P < 0.01$) in the probiotic treatment (43.7 ± 6.2% CD4 cell mm^{-2}) than in the control (25.4 ± 3.5% CD4 cell mm^{-2}) 7 (Fig. 2).

DISCUSSION

The use of *Bacillus* species as a probiotic in aquaculture is common (Gatesoupe, 1999). For example, it has been shown that *Bacillus subtilis* can improve the immune conditions, growth and survival of *Oreochromis niloticus* (Aly *et al.*, 2008), *Ictalurus punctatus* (Queiroz & Boyd, 1998) and shrimp *Penaeus monodon* (Rengpipat *et al.*, 2000).

Due to the addition of commercial *Bacillus* strains into probiotic treatment, total spore-forming bacteria counts were higher in this group. The water exchange rate in the RAS tanks is increased as fish grow larger to maintain homogeneous fish and live feed distribution (Holt *et al.*, 2007). This practice makes water recirculate faster in the system filters, mechanically removing probiotics, and it increases the flow of water into the UV sterilizer. In open aquaculture systems such as ponds and tanks, probiotics are added after each water exchange. However, in RAS the appropriate frequency of probiotics addition has not been determined and would likely differ from that recommended by the manufacturers for open systems (Garrido-Pereira *et al.,* 2013). The presence of spore-forming bacteria in control may be explained by the presence of different bacteria sporulating in marine water (Tarasenko *et al.,* 2012).

Table 1. Survival (SR), final weight (FW), total ammonia (TAN), nitrite (NO$_2$), nitrate (NO$_3$), total *Vibrio* (TV) and total spore-forming bacteria (TSB) of cobia larvae reared for 26 days. Values represent mean ± standard deviation of two treatments from triplicate tanks. Values in the same column with different superscript are significantly different from each other ($P < 0.05$).

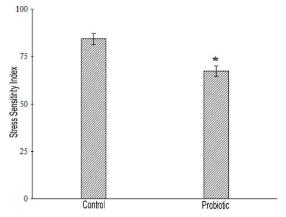| Treatment	SR (%)	FW (mg)	TAN (mg L^{-1})	NO$_2$ (mg L^{-1})	NO$_3$ (mg L^{-1})	TV (CFU mL^{-1})	TSB (CFU mL^{-1})
Probiotic	14.9 ± 2.4[a]	170 ± 10[a]	0.13 ± 0.04[a]	0.05 ± 0.03[a]	2.99 ± 0.81[a]	3366 ± 522[a]	901 ± 88 [a]
Control	15.1 ± 0.9 [a]	160 ± 10[a]	0.12 ± 0.03[a]	0.04 ± 0.02[a]	2.93 ± 0.70[a]	4964 ± 902[a]	61 ± 24 [b]

Figure 1. Stress Sensitivity Index of cobia larvae reared with or without probiotic at the end of experimental period. Vertical bars represent one standard deviation and asterisk shows statistically significant difference ($P < 0.01$).

Figure 2. CD3 and CD4 expression (%/mm^2) in thymus of cobia larvae reared with or without probiotic. Vertical bars represent one standard deviation and asterisk shows significantly statistic difference ($P < 0.01$).

Vibrio spp. are normally present in larval gut flora and in the live feed, and they usually cause disease under sub-optimal culture conditions (Decamp *et al.,* 2008). Several authors (Moriarty, 1998; Gatesoupe, 1999; Chen & Chen, 2001; Vaseeharan & Ramasamy, 2003) reported an inhibitory effect of circulating *Bacillus* on *Vibrio* loads. In our trial *Vibrio* counts were apparently not affected by probiotic bacteria, but fish showed no sign of vibriosis or other diseases.

Larvae reared with probiotic during this trial showed greater resistance to the stress test, suggesting that larvae supplemented with *Bacillus* spp. have better quality than those reared without probiotic. According to Dhert *et al.* (1992), when organisms are exposed to different treatments they will first undergo some physiological changes, which will inevitably affect their resistance, before the impact on growth and survival may be noticed. Survival is commonly used as a sole indicator in determining larval quality, however it can be influenced by many parameters and it does not reveal the actual condition of the fish.

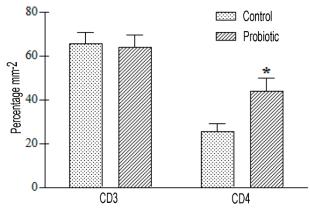Immuno histochemistry analysis confirmed CD3 and CD4 thymocytes at 26 dph in cobia, suggesting that critical events of differentiation of T lymphocytes could occur before weaning in this species. The thymus plays a pivotal role in the adaptive immune system and the CD3 complex is a co-receptor that serves as a marker for general T cell identification in fish (Øvergård *et al.*, 2009). One of the major subclasses of T cells is CD4-expressing cells called T helper, which is originally a CD3-expressing lymphocyte that receives a CD4 receptor during the process of maturation (Laing *et al.,* 2006). This type of lymphocyte stimulates the secretion of cytokines that produce antibody responses or lead to macrophage activation (Buonocore *et al.,* 2008). In present work there was no increase in the number of T lymphocytes in general, but there was an increase of T helper type in the probiotic treatment. This demonstrates the stimulation of T cells differentiation into T helper achieved by *Bacillus* spp. in the probiotic treatment, since the expression of CD4 was higher in this group.

Olafsen (2001), Picchietti *et al.* (2007) and Guzmán-Villanueva *et al.* (2014) reported the use of live feed cultured with selected bacterial strains to improve immunity, growth and survival. The ingestion of live feed containing probiotics may have been

crucial in this trial, since RAS have equipments that can hamper the positive effects of probiotic bacteria added into the water (Garrido-Pereira *et al.,* 2013). Further investigations must be conducted to evaluate optimal probiotic doses for RAS.

In conclusion, the probiotic bacteria (*B. subtilis*, *B. pumilus* and *B. licheniformis*) tested in this trial stimulated the immunological system by T-cells differentiation in cobia larvae and improved their salinity stress tolerance although it had no noticeable effect on survival or growth.

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