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Effects of a mix of *Bacillus* sp. as a potential probiotic for Florida pompano, common snook and red drum larvae performances and digestive enzyme activities

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Abstract :

This study examined the effect of a commercial mix of *Bacillus* sp. on survival, growth and digestive enzyme activities of Florida pompano, red drum and common snook. Larvae were fed either live feed enriched with Algamac 3050 (Control), Algamac 3050 and probiotics (PB), or the previous diet combined with a daily addition of probiotics to the tank water (PB+). Survival was not affected by the treatments for any of the species. At the end of the pompano and snook trial, standard lengths of larvae from the PB and PB+ treatments were significantly greater than for the control larvae. Microbiological analyses were performed at the end of the pompano trial, and numbers of presumptive Vibrio were not a concern in the system. For both pompano and snook, trypsin-specific activity was higher in PB and PB+ larvae compared with the control larvae. Similarly, alkaline phosphatase activity was higher for the pompano larvae fed the PB and PB+ treatments and for the snook larvae fed the PB+ treatment compared with the control larvae. This experiment suggests that a mix of *Bacillus* sp. can promote growth through an early maturation of the digestive system during the early larval stages of pompano and snook.

Keywords : *Centropomus undecimalis*, digestive enzyme, fish larvae, probiotic, *Sciaenops ocellatus*, *Trachinotus carolinus*

INTRODUCTION

Marine fish larvae undergo major physiological and morphological changes during the first weeks of their lives (Péres *et al.* 1997). Inadequate nutrition and larval rearing conditions during this crucial transitional period adversely affect the development and the future success of the larvae (Yúfera and Darias 2007). Determining optimal rearing protocols has proven difficult in marine fish due to the small size and fragility of the larvae, therefore, the mass production of robust juveniles remains a challenge for most species (Hamre *et al.* 2013). The external environment has a major impact on marine fish gastrointestinal microflora as osmoregulation requires constant ingestion of the surrounding water (Gatesoupe 1999). Since the digestive tract of fish larvae is sterile at hatching, the initial colonization depends on the environment and live feed ingested (Grisez *et al.* 1997). The initial microflora established at the larval stage seems to persist after metamorphosis, it is therefore thought that providing probiotics as soon as possible after hatching can have beneficial effects (Ringø and Vadstein 1998). In addition, the larvae immune system is immature and relies on nonspecific defense mechanisms, thus a healthy microflora constitutes a crucial primary barrier to which probiotics can, most likely, effectively contribute (Hansen and Olafsen 1999).

Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (Aureli *et al.* 2011). In humans, Hooper *et al*. (2001) demonstrated that probiotics can modulate the expression of genes involved in nutrient absorption, mucosal barrier fortification and postnatal intestinal maturation. Bacteria communicate via the use of quorum-sensing molecules, which regulate gene expression mainly when the population has reached a high cell density (Williams *et al.* 2007). *B. subtilis* was found to produce a quorum-sensing pentapeptide, the competence and sporulation-stimulating factor (CSF), which activates two cellular survival pathways (protein kinase B and p38 mitogen-activated protein kinase) and induces the expression of the heat shock protein (e.g. Hsp27) in intestinal

epithelial cells (Fujiya *et al.* 2007). Hsp protect cells against various stresses and this mechanism is highly conserved throughout evolution and across species (Parsell and Lindquist 1993). When over expressed, Hsp increase intestinal epithelial cells viability and protect from oxidative injury, contributing to intestinal homeostasis (Tao *et al.* 2006).

Probiotics have various modes of actions including the competitive exclusion of pathogenic bacteria (Moriarty 1997, Gomez-Gil *et al.* 2000, Chythanya *et al.* 2002, Balcázar *et al.* 2004, Vine *et al.* 2004), the improvement of water quality (Moriarty 1997), the enhancement of the immune system (Gatesoupe 1999, Balcázar *et al.* 2004, Picchietti *et al.* 2009, Zhou *et al.* 2010) and the stimulation of the digestive system (Suzer *et al.* 2008, Lazado *et al.* 2012).

Among the probiotics, *Bacillus* is of particular interest as it is a spore forming bacteria (Cutting 2011). Spore production is triggered by nutrient depletion in the bacterial environment, allowing for long-term survival in conditions inadequate to vegetative bacteria. Even though spores are dehydrated and have an inactive metabolism, they are able to monitor the environment (Nicholson *et al.* 2000). Under appropriate conditions, germination occurs by allowing water to penetrate the spore and vegetative growth resumes (Moir 2006). The spore surface layer confers outstanding resistance to extreme physical and chemical stress (Henriques and Moran 2007). Spores are heat stable and can survive the low pH of the gastric barrier (Spinosa *et al.* 2000). Therefore, they can be stored at room temperature in a desiccated form for a long period of time and all of the administrated spores will reach the intestinal tract (Cutting 2011). In addition, production cost is low, making *Bacillus* particularly valuable for use in aquaculture production (Wang *et al.* 2008).

In fish, the administration of *Bacillus* was found to positively influence expression of genes involved in growth metabolism and animal welfare in sea bream (Avella *et al.* 2010). In the same species, *Bacillus* was shown to increase the expression of occludin, a trans-membrane component of tight junctions in the intestine, suggesting an improvement of cell junction

integrity between enterocytes (Cerezuela *et al.* 2013). On white shrimp *Penaeus vannamei*, a *Bacillus coagulants* supplementation improved growth performances and enhanced the immune response through antibacterial activity as well as an increase in phenoloxidase, superoxide dismutase and peroxidase activities (Wang and Gu 2010). In addition, studies have demonstrated, but not explained, the ability for *Bacillus* to increase resistance to vibrio in black tiger shrimp *Penaeus monodon* (Vaseeharan and Ramasamy 2003), common snook, *Centropomus undecimalis* (Kennedy *et al.* 1998) and sea bass *Dicentrarchus labrax* (Touraki *et al.* 2012); increase growth and stimulate the digestive system in Indian white shrimp *Fenneropenaeus indicus* (Ziaei-Nejad *et al.* 2006), Pacific white shrimp *Penaeus vannamei* (Wang 2007), Japanese flounder *Paralichthys olivaceus* (Ye *et al.* 2011), orange-spotted grouper *Epinephelus coioides* (Sun *et al.* 2013), rohu *Labeo rohita* (Mohapatra *et al.* 2012) and common carp *Cyprinus carpio* (Wang and Zirong 2006); promote growth and improve tolerance to rearing conditions in sea bream *Sparus aurata* (Avella *et al.* 2010).

The present study aimed to test the effects of a commercial mix of *Bacillus* (Sanolife MIC-F, INVE Technologies, Belgium) on the growth and digestive enzyme activities in early larval stages of some of Florida's high-value marine food fish (Florida pompano *Trachinotus carolinus* and red drum *Sciaenops ocellatus*) and stock enhancement species (common snook *Centropomus undecimalis*).

MATERIALS AND METHODS

Experimental animals

Snook and pompano eggs were obtained from broodstock captured on the southwest Florida coast and held at the Mote Marine Laboratory Aquaculture Research Park in Sarasota, Florida. Broodstock were conditioned through photothermal regimes in tanks (25 m^3 for pompano, 45 m^3 for snook) equipped with recirculating filtration systems. Spawning was induced by implanting mature pompano females with 50 $\mu g.kg^{-1}$ of sGnRHa (Ovaplant®) and mature snook females with 50 $\mu g.kg^{-1}$ of sGnRHa from the Institute of Marine and Environmental Technology of the University of Maryland. Red drum eggs were received from captive broodstock held at the Florida Fish and Wildlife Conservation Commission Stock Enhancement Research Facility.

Eggs for each species were incubated in a 100 L hatching tank with aeration and an upwelling water circulation from a $3m^3$ system with UV and bio-filtration. Fertilization and hatching rates were respectively 54.5 % and 73.2 % for pompano and 83.5 % and 85.2 % for snook. The fertilization rate of the red drum eggs was unknown while the hatching rate reached 90.2 %.

Experimental set up and treatments

The experimental set up included three identical independent systems. Each system was composed of four 100L tanks with water recirculating from the tanks to a biofilter and back to the tanks via a UV light. Each independent system was assigned a treatment to avoid probiotic cross contamination.

After hatching, larvae were volumetrically counted and transferred to the experimental tanks at 100 larvae per liter for pompano and red drum, and 200 larvae per liter for snook according to standard procedures at the research park. For all species, photoperiod was maintained at

12h dark: 12h light, salinity at 35 ± 1 $g.L^{-1}$, temperature at 27 ± 1 °C, pH at 8 ± 0.5, dissolved oxygen at 6 ± 2 $mg.L^{-1}$. From 2 days post hatch (DPH), rotifers were fed twice a day at 5 rotifers per mL. In addition, a microdiet (Gemma, Skretting, France) was delivered twice a day in between rotifer feeding.

Trials were stopped at the end of the rotifer-feeding period for pompano (9 DPH) and snook (12 DPH). The red drum trial was extended up to 21 DPH due to the lack of significant difference in growth at the end of the rotifer-feeding period (10 DPH). In this case, rotifer density was decreased to 3 per ml at 7 DPH and the fish weaned onto the microdiet from 10 DPH.

Three treatments were tested in quadruplicate for all trials. The first treatment (control) was rotifers enriched with Algamac 3050 (Aquafauna Bio-Marine Inc, USA). The second treatment (PB) was rotifers enriched with Algamac 3050 and a commercial mix of *Bacillus* spp. (0.5 g per liter of enrichment according to manufacturer's recommendations, concentration of bacteria: minimum $1x10^{10}$ CFU/g). The third treatment (PB+) was the second treatment, with additional probiotics (5 $g.m^{-3}$, according to manufacturer's recommendations) added daily directly to the tank water.

Sampling

Larvae growth was monitored through standard length measurement of 10 larvae from each tank (40 per treatment) at 1, 5 and 9 DPH for pompano; 1, 5, 9, and 12 DPH for snook; 1, 7, 14 and 21 DPH for red drum. Pompano larvae body depth was also recorded in consideration to the particular short, deep and compressed pompano body shape. At the end of the trials, 50 larvae from each tank (200 per treatment) were preserved at -70°C for enzyme analysis. In addition, at the end of the pompano trial, 25 larvae from each tank (100 per treatment) were preserved at 4°C for bacterial analyses performed the following day.

Enzyme and bacterial analyses

Enzyme analyses were performed at the Functional Physiology of Marine Organisms unit at Ifremer in Brest, France. Larvae from each tank were pooled and homogenized prior to all analyses. Trypsin, amylase, alkaline phosphatase (AP) and leucine–alanine peptidase (Leu-ala) activities were assayed according to Holm et al. (1988), Métais and Bieth (1968), Bessey et al. (1946) and Nicholson and Kim 1975, respectively. Enzyme activity results are expressed as specific activities, i.e. $U.mg^{-1}$ protein. Protein was determined by the Bradford procedure (Bradford 1976). Due to technical difficulties, red drum larvae sampled at the end of the rotifer feeding period (7 dph) could not be processed and enzyme analyses were performed at 21 dph only, therefore, results from the red drum trial are presented separately in table 2.

Bacterial analyses were performed at the Mote Marine Laboratory, Center for Marine Microbiology. Larvae from each tank were pooled and rinsed three times with sterile seawater then ground using a PowerSoil® DNA isolation kit (MO-BIO Laboratories, Inc., USA). Serial dilutions of the homogenates were then plated on marine agar (promoting the growth of all marine heterotrophs) and TCBS (medium selective of *Vibrio* sp.) media. The petri dishes were incubated at 22 °C and the number of colony-forming units were counted 48 hours after plating.

Probiotic strains identification

One gram of the commercial mix was diluted in 99 ml of Phosphate-Buffered Saline (PBS) and mixed thoroughly. An inoculating loopful of the suspension was then plated following the quadrant method on Trypticase Soy Agar (TSA) media. The plate was incubated inverted at 37 °C overnight. Colonies showing distinct morphologies were sub-cultured on TSA media

following the same method. Isolated strains were sent for 16S rDNA sequencing identification to Accugenix, Inc. (Newark, DE, USA).

Statistical analysis

Statistical analyses were performed using MINITAB® version 16.0. Normality and homogeneity of variance were confirmed using Kolmogorov–Smirnov test. Growth and body depth data were analyzed using a General Linear Model (GLM) with all time and treatment interactions being analyzed and significant differences tested by a Tukey post-hoc test with 95 % confidence. Bacterial counts and enzyme activities were compared by a one-way ANOVA followed by a Tukey post hoc test with 95 % confidence. Survival data was arcsine square root transformed before a one-way ANOVA followed by a Tukey post-hoc test with 95 % confidence.

RESULTS

***Bacillus* strains identification**

Three *Bacillus* strains were isolated from the commercial mix. The 16S rDNA sequence-based identified the following species: *Bacillus licheniformis*, *Bacillus amyloliquefaciens plantarum/methylotrophicus* and *Bacillus pumilus/safensis*. In the two later cases, the strain matched two closely related species that cannot be differentiated by 16S rDNA (Fig. 1).

Survival

No significant difference in survival from hatching to the end of the trial was observed between treatments regardless of the species. However, survival was significantly higher in pompano (7.6 ± 1.9 %) and red drum (9.9 ± 0.8 %) compared to snook (2.4 ± 0.7 %) (Fig.2).

During the snook trial, poor survival led to the termination of one PB tank at 7 DPH, as well as one Control and one PB+ tank at DPH 9.

Growth

At the end of the pompano trial, PB and PB+ larvae had significantly greater standard length (Fig. 3A) and body depth (Fig. 3B) than the control larvae with 4.34 ± 0.10, 4.22 ± 0.07 and 3.89 ± 0.09 mm, respectively for standard length and 0.88 ± 0.01, 0.83 ± 0.03 and 0.66 ± 0.01, respectively for body depth. The same was true for snook larvae standard length with 3.69 ± 0.02, 3.60 ± 0.03 and 3.29 ± 0.03 mm, respectively for PB, PB+ and control larvae (Fig. 3C). However, no significant difference was observed for body depth with an average of 0.71 ± 0.20 mm at the end of the experiment (Fig. 3D). At the end of the red drum trial, no difference was observed between treatments with an average of 5.44 ± 0.07 mm (Table 1).

Bacterial analyses

Results from the microbiological analyses on the pompano larvae showed significantly higher counts of colony-forming units (CFU) per larvae on the marine agar media for the larvae fed the probiotics supplementation ($38.10^3 \pm 8.10^3$ CFU for PB and $18.10^3 \pm 22.10^3$ CFU for PB+) compared to the control larvae ($10^3 \pm 0.6.10^3$ CFU). Numbers of presumptive *Vibrio* on the TCBS media were low and not significantly different between treatments with an average of 0.06 ± 10^3 CFU per larvae (Table 2).

Enzyme activities

For both pompano and snook, trypsin specific activities at the end of the trial were significantly higher in larvae fed the PB and the PB+ treatments compared to the control larvae. Trypsin activities of snook larvae from the PB and PB+ treatments were respectively

37.3 % and 29.6 % higher than that of control larvae, while trypsin activities of pompano larvae from the same treatments were respectively 45.1 % and 46.8 % higher than that of control larvae (Fig. 4a).

Difference in amylase activity was only observed for snook larvae, with activities of larvae from the PB+ treatment 65.2 % higher than that of control larvae (Fig. 4b).

AP activities of pompano larvae from the PB and PB+ treatments and snook larvae from the PB+ treatment were higher than that of control larvae. AP activity of snook larvae from the PB+ treatments was 27.1 % higher than that of control larvae while AP activities of pompano larvae from the PB and PB+ treatments were respectively 27.9 % and 28.0 % higher than that of control larvae. For all treatments, AP activities of pompano larvae were significantly higher than that of snook larvae (Fig. 4c).

For both snook and pompano, no significant differences were observed in Leu-ala activities between treatments or between species (Fig. 4d).

No significant differences were observed in the activities of the enzymes tested for red drum at the end of the trial (Table 1).

DISCUSSION

This series of experiments clearly suggested a beneficial effect of *Bacillus* probiotic on growth and digestive enzyme activity of Florida pompano and Common snook larvae when supplemented during the early larval stages. Similar results have been observed with Indian white shrimp (Ziaei-Nejad *et al.* 2006), common carp (Wang and Zirong 2006), Pacific white shrimp (Wang 2007), Japanese flounder (Ye *et al.* 2011), rohu (Mohapatra *et al.* 2012) and orange-spotted grouper (Sun *et al.* 2013).

The three strains identified in the commercial mix are species closely related to *Bacillus subtilis* however, they differ metabolically and secrete different enzymes (Priest *et al.* 1987). *B. pumilus* isolated from the gut of rohu fingerlings was found to produce amylase and cellulase (Ghosh *et al.* 2002) and it demonstrated strong inhibition against several strains of *Vibrio* sp. when isolated from the gut of black tiger shrimp (Hill *et al.* 2009). *B. licheniformis* has been reported to have antiviral properties through the induction of cytokines (Arena *et al.* 2006) and to produce an antimicrobial peptide with a broad inhibitory spectrum (Cladera-Olivera *et al.* 2004). In addition it was shown to produce phytase when isolated from several freshwater cultured fish (Dan and Ray 2013). *B. amyloliquefaciens* is closely related to *B. subtilis* and used to be given a subspecies status (*B. subtilis* subsp. *amyloliquefaciens)* before additional studies demonstrated the numerous physiological and biochemical specificities of *B. amyloliquefaciens* (Priest *et al.* 1987). One of the main differences between the two species is the ability of *B. amyloliquefaciens* to produce more extracellular enzyme than *B. subtilis*, including between 50 and 150 times more α-amylase (Welker and Campbell 1967, Priest *et al.* 1987). In fish, *B. amyloliquefaciens* was found to have an inhibitory effect on pathogenic *Aeromonas hydrophila* associated with the eel *Anguilla anguilla* (Cao *et al.* 2011), and improved growth, feed conversion ratio and immunological parameters in Nile

tilapia *Oreochromis niloticus* (Ridha and Azad 2012). The addition of either *B. subtilis*, *B. licheniformis* or *B. pumilus* to the diet of olive flounder *Paralichthys olivaceus*, led to different effects with *B. subtilis* increasing growth, *B. subtilis* and *B. pumilus* increasing survival rate, and *B. pumilus* and *B. licheniformis* increasing superoxide dismutase activity and disease resistance (Cha *et al.* 2013). These results highlight the interest of supplementing several strains of probiotics simultaneously. Nonetheless, it is likely that the inclusion rate of each strain will impact on the final effect of the product and therefore manufacturers should communicate not only on the qualitative characteristics of their product, but also on the quantitative characteristics.

Several modes of action are proposed to explain the positive effect of probiotics, including antagonism towards pathogens, competition for adhesion sites and competitions for nutrients (Ray *et al.* 2012). However, the microbiological analyses on the pompano larvae at the end of the trial did not show high vibrio counts in any of the treatments. Therefore, it was assumed that in the experimental system used for these trials, pathogenic bacteria were not a major issue and no microbiological analyses were performed for the other species. Counts of heterotrophic bacteria were significantly higher in the PB and PB+ treatments compared to the control, confirming the presence of the probiotics in the gut of the larvae.

Probiotics can also act on the digestive system of their host. Poorly developed at hatching, the digestive system of marine fish larvae matures progressively, evolving from an intracellular mode of digestion via pinocytosis, to an adult mode of digestion involving membrane transport with the development of the brush border membrane (Govoni *et al.* 1986). Alkaline phosphatase, an enzyme mainly located in the brush border membrane of enterocytes, is therefore a good indicator of intestinal development (Cahu and Zambonino-Infante 1995). Simultaneously to the intestine maturation, the functional maturation of the pancreas occurs, with an activity increase of proteolytic enzymes, including trypsin, and a decrease in the

carbolytic enzyme amylase (Cahu and Zambonino-Infante 1994). In this study, results from the enzyme analyses showed an increase in trypsin specific activity for the pompano and snook larvae fed the probiotic supplementation. In addition, the specific activity of alkaline phosphatase was significantly higher for pompano larvae fed the PB and PB+ treatments and for snook larvae fed the PB+ treatment, suggesting an early maturation of the digestive system. The higher amylase specific activity in snook larvae fed the PB+ treatment might be due to a higher capacity of snook to utilize carbohydrates, which could be stimulated by the important extracellular amylase production by *B. amyloliquefaciens.* Very little is known about the natural diet of the early larval stage of snook and pompano and more research is needed to understand such variations.

It was hypothesized that the increase in enzyme activities observed with the use of probiotics could be due to the exoenzymes produced by the bacteria (Bairagi *et al.* 2002, Balcázar *et al.* 2006). Nonetheless, Ziaei-Nejad et al. (2006) demonstrated that the proportion of enzyme synthesized by the probiotics could only contribute to a very small amount of the total enzyme activity of the gut and suggested that instead, the probiotics stimulate the production of endogenous enzymes.

In the present study, the increased level of maturation of the enterocytes of the snook and pompano larvae fed the probiotic supplementation suggested an increased absorptive capacity of the brush-border membrane leading to more efficient feed utilization and better growth.

Pompano and snook larvae are not as robust as red drum larvae and seem to benefit more from the probiotic supplementation. However, no detrimental effect was observed for the red drum larvae and the probiotics might have influenced factors other than growth and digestive enzyme activity, such as disease and stress resistance, intestinal epithelium structure or general welfare as discussed above. In addition, a longer trial period might have revealed some differences in growth as Ridha and Azad (2012) did not observe any growth differences

after 99 days when juvenile Nile tilapia were fed a diet enriched with *Bacillus* but observed differences 61 day after the end of the treatment compared to the control treatment. Moreover, a higher level of probiotic supplementation might be necessary. Indeed, Merrifield et al. (2010) showed that high intestinal levels of *B. subtilis* and *B. licheniformis* (>80 %) are required to improve rainbow trout growth performance and feed utilization.

No difference in survival was observed between treatments for all species although an increase in survival would be expected alongside the advanced digestive system maturation and improved growth, especially for snook where survival is very low. Many factors participate in the survival of young fish larvae and snook being a relatively new species in aquaculture, many rearing aspects still need to be explored and improved. Even though the probiotic supplementation did not increase survival it is likely that larvae with improved growth and digestive capabilities will be more robust and a difference in survival might be observed after critical life events such as metamorphosis and weaning.

In conclusion, these experiments demonstrated positive effects from the mix of *Bacillus* on the development of pompano and snook larvae through an early maturation of the digestive tract. To obtain optimal effects, a supplementation through both the live feed and the tank water seems recommended. Further research is needed to better understand the mode of action of probiotics and the mechanisms involved during the ontogeny of the digestive system.

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Figure headlines

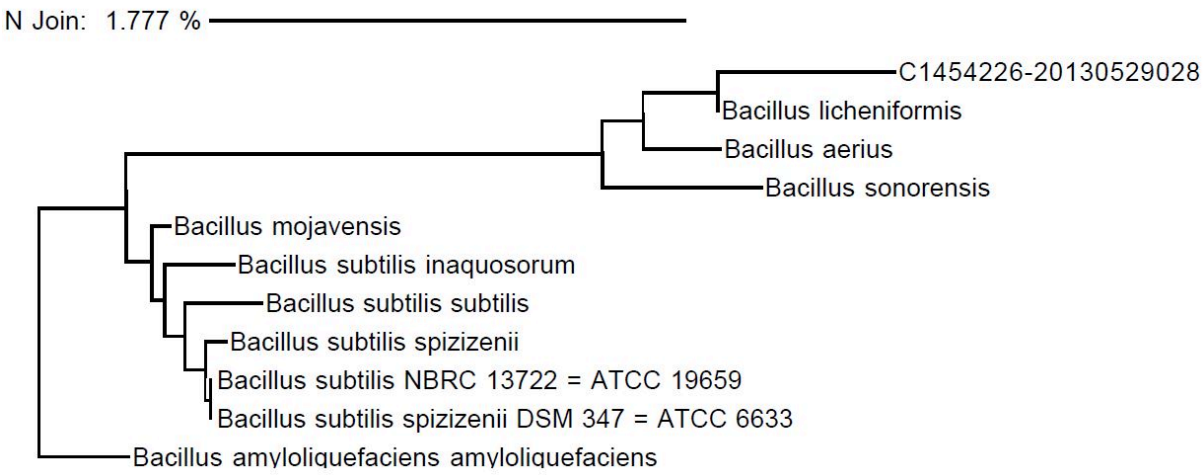
Figure 1: Phylogenetic trees constructed using the neighbor-joining method for each of the isolated strains. The N join scale bar provides a horizontal distance scale.

Figure 2. Survival from hatching for snook, pompano and red drum at the end of the trial. Mean ± standard error (n=3 for snook, n=4 for pompano and red rum). Letters indicate significant differences.

Figure 3: Body depth and standard length of pompano and snook larvae at the end of the trial. Mean ± standard error (n=4 for pompano, n=4 for snook at 1 and 5 DPH then n=3, 10 larvae per tank and time point). Letters indicate significant differences between times and treatments.

Figure 4: Specific activities ($U.mg^{-1}$ of protein) of trypsin, amylase, alkaline phosphatase (AP) and leucine-alanine peptidase (leu-ala) of snook and pompano larvae during the trial. Mean ± standard error (n=3 and 4 respectively for snook and pompano, 50 larvae per tank). Letters indicate significant differences between treatments for snook (uppercase letters), pompano (italic uppercase letters) and between treatment and species (lower case letters).

A)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C1454226-20130529028
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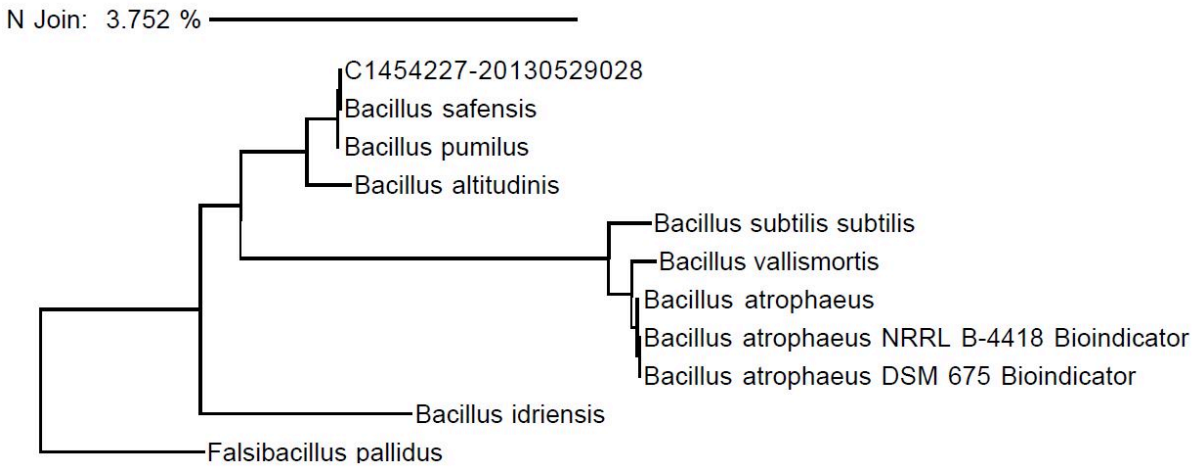
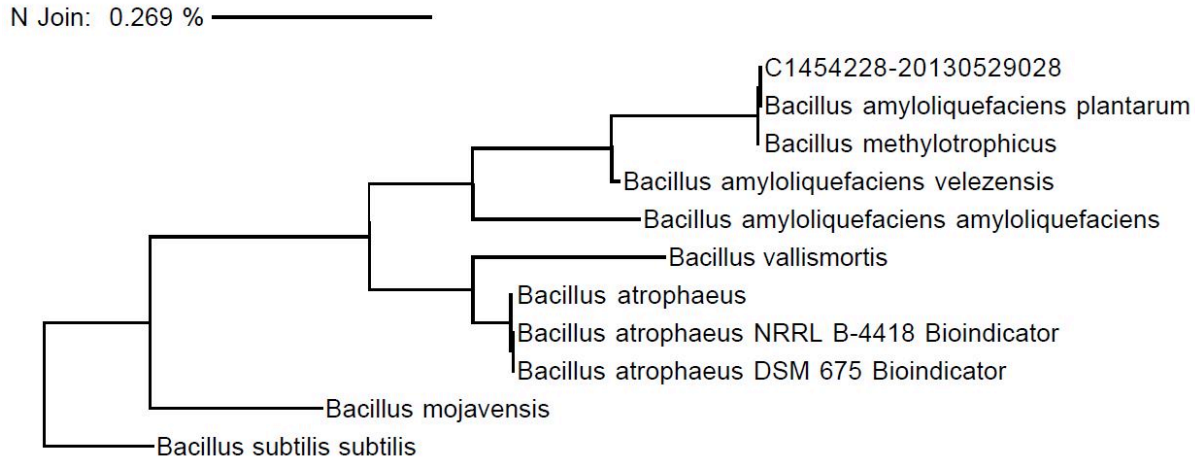
Bacillus aerius
Bacillus sonorensis
Bacillus mojavensis
Bacillus subtilis inaquosorum
Bacillus subtilis subtilis
Bacillus subtilis spizizenii
Bacillus subtilis NBRC 13722 = ATCC 19659
Bacillus subtilis spizizenii DSM 347 = ATCC 6633
Bacillus amyloliquefaciens amyloliquefaciens

B)


N Join: 3.752 %
C1454227-20130529028
Bacillus safensis
Bacillus pumilus
Bacillus altitudinis
Bacillus subtilis subtilis
Bacillus vallismortis
Bacillus atrophaeus
Bacillus atrophaeus NRRL B-4418 Bioindicator
Bacillus atrophaeus DSM 675 Bioindicator
Bacillus idriensis
Falsibacillus pallidus

C)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C1454228-20130529028
Bacillus amyloliquefaciens plantarum
Bacillus methylotrophicus
Bacillus amyloliquefaciens velezensis
Bacillus amyloliquefaciens amyloliquefaciens
Bacillus vallismortis
Bacillus atrophaeus
Bacillus atrophaeus NRRL B-4418 Bioindicator
Bacillus atrophaeus DSM 675 Bioindicator
Bacillus mojavensis
Bacillus subtilis subtilis

Figure 1

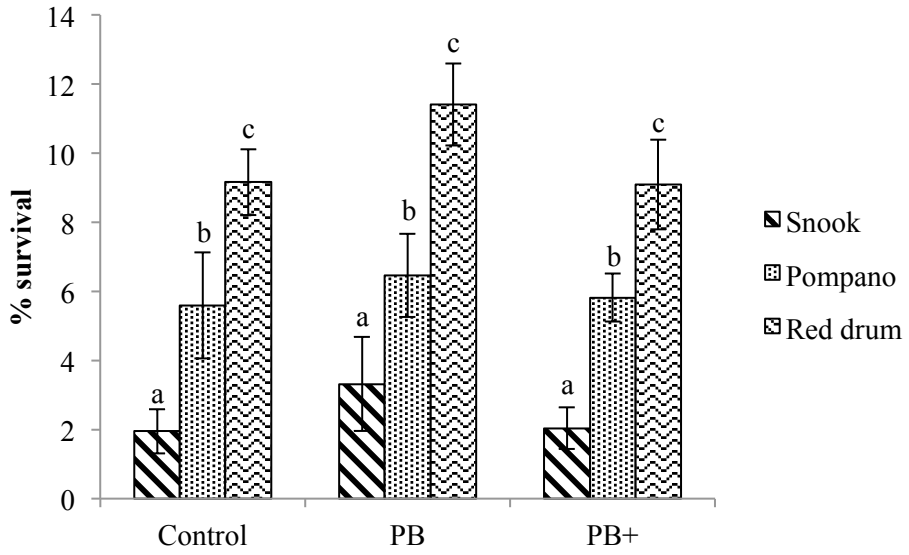
14
12
10
8
6
4
2
0
% survival
a
b
c
a
b
c
a
b
c
Control
PB
PB+
Snook
Pompano
Red drum

Figure 2.

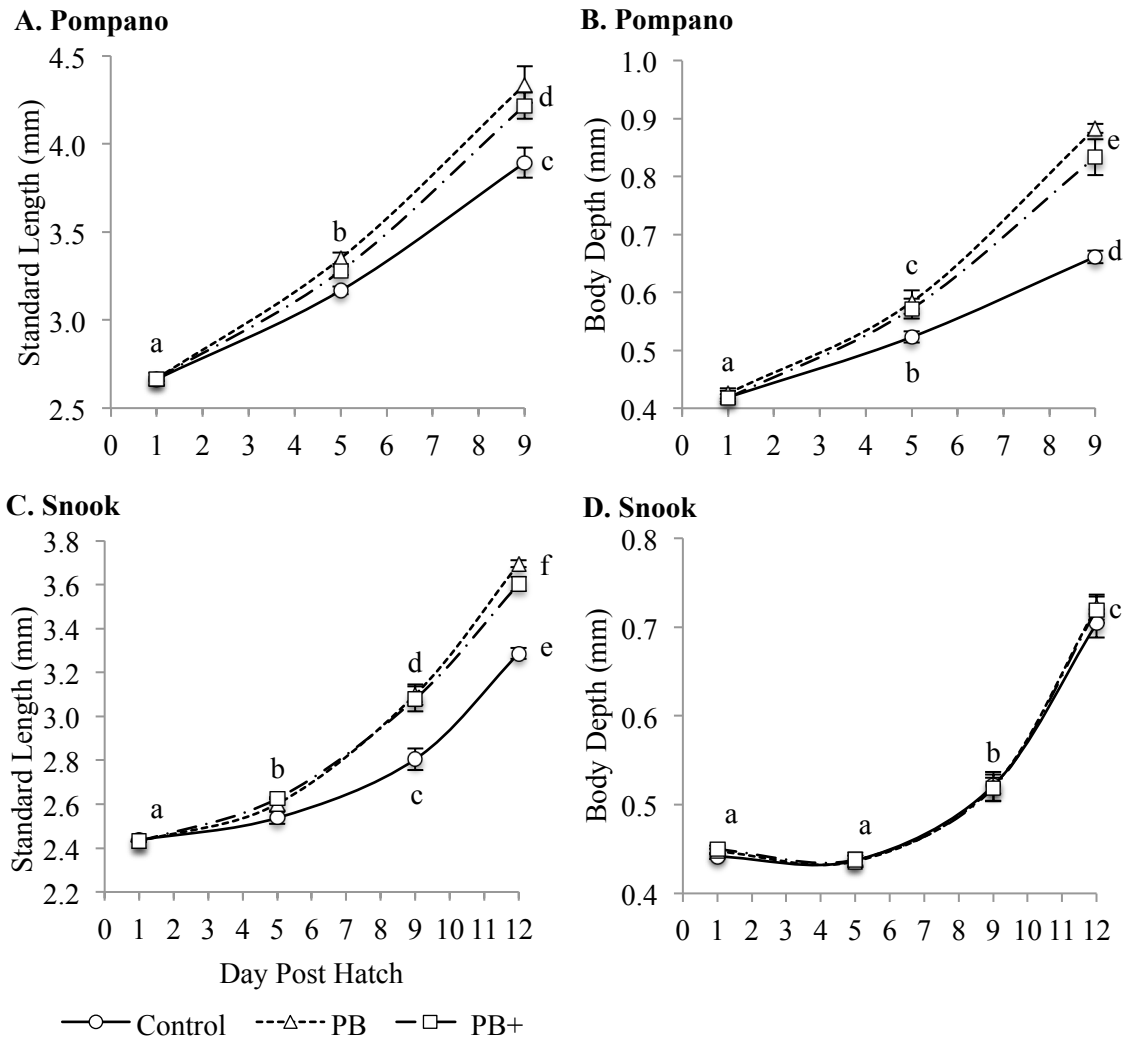
A. Pompano
B. Pompano
C. Snook
D. Snook
Standard Length (mm)
Body Depth (mm)
Day Post Hatch
Control
PB
PB+

Figure 3.

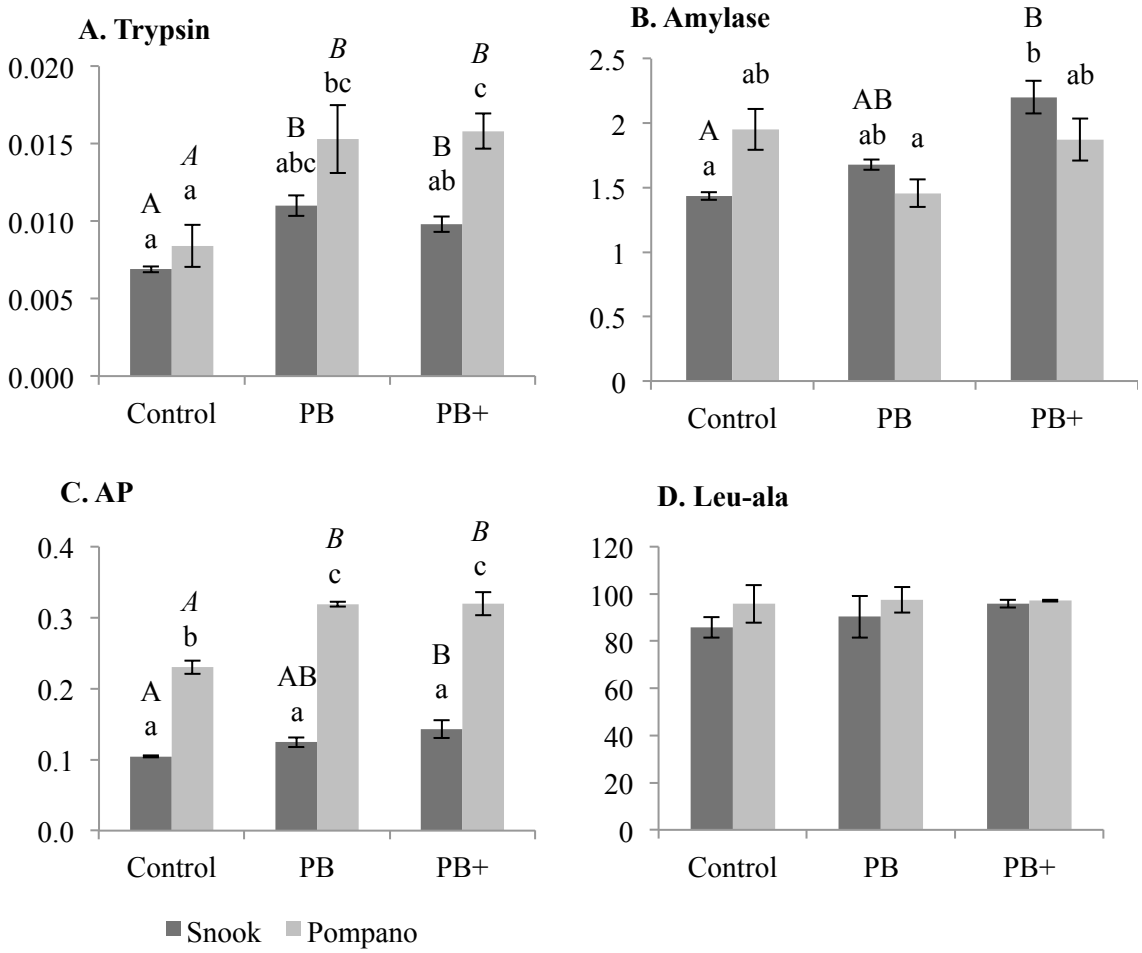
A. Trypsin
0.020
0.015
0.010
0.005
0.000
A
a
A
a
B
abc
B
bc
B
ab
B
c
Control
PB
PB+
B. Amylase
2.5
2
1.5
1
0.5
0
A
a
ab
AB
ab
a
B
b
ab
Control
PB
PB+
C. AP
0.4
0.3
0.2
0.1
0.0
A
a
A
b
AB
a
B
c
B
a
B
c
Control
PB
PB+
D. Leu-ala
120
100
80
60
40
20
0
Control
PB
PB+
Snook
Pompano

Figure 4.

Table 1. Effects of a probiotic supplementation (PB and PB+) on standard length (mm) and specific activities ($U.mg^{-1}$ of protein) of trypsin, amylase, alkaline phosphatase (AP) and leucine-alanine peptidase (leu-ala) of red drum larvae. Mean ± standard error (n=4 tanks, 50 larvae per tank). No statistical differences were observed.

	Control	PB	PB+
Growth			
1 DPH	2.82 ± 0.01	2.81 ± 0.02	2.79 ± 0.01
7 DPH	3.51 ± 0.02	3.55 ± 0.01	3.58 ± 0.01
14 DPH	4.67 ± 0.01	4.48 ± 0.03	4.58 ± 0.02
21 DPH	5.54 ± 0.03	5.45 ± 0.08	5.32 ± 0.08
Specific activity at 21 DPH			
Trypsin	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Amylase	0.56 ± 0.04	0.42 ± 0.07	0.51 ± 0.03
AP	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.00
Leu-ala	145.8 ± 4.2	118.9 ± 3.2	143.1 ± 5.8

Table 2. Number of colony-forming units ($x10^3 \pm$ standard error of the mean) per pompano larvae (n=4 tank, 10 larvae per tank) fed without probiotic supplementation (Control), with probiotic supplementation in the live food (PB) or with probiotic supplementation in the live food and tank water (PB+). Superscript letters indicate significant differences within the same column (Tukey test, $p<0.05$).

	Marine Agar	TCBS
Control	1.0 ± 0.6^{a}	0.01 ± 0.00^{a}
PB	38 ± 8^{b}	0.15 ± 0.01^{a}
PB+	18 ± 22^{b}	0.02 ± 0.01^{a}