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Effects of Probiotics on the Aquaculture Performance of Cobia (Rachycentron canadum) and Yellowfin tuna (Thunnus albacares)

Juan Felipe Sierra De la Rosa
University of Miami, juanfelipesierradelarosa@gmail.com

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EFFECTS OF PROBIOTICS ON THE AQUACULTURE PERFORMANCE OF COBIA
(Rachycentron canadum) AND YELLOWFIN TUNA (Thunnus albacares)

By
Juan Felipe Sierra-De la Rosa

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
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EFFECTS OF PROBIOTICS ON THE AQUACULTURE
PERFORMANCE OF COBIA (Rachycentron canadum) AND
YELLOWFIN TUNA (Thunnus albacares)

Juan Felipe Sierra-De la Rosa

Approved:

________________  ____________________
Daniel D. Benetti, Ph.D.                       Marjorie Oleksiak, Ph.D.
Professor of Marine Ecosystems and Society     Professor of Marine Biology
                                              and Ecology

________________  ____________________
Larry E. Brand, Ph.D.                          Guillermo Prado, Ph.D.
Professor of Marine Biology and Ecology        Dean of the Graduate School

________________
Daniel Margulies, Ph.D.
Senior Scientist, Inter-American
Tropical Tuna Commission, La Jolla, California
Effects of Probiotics on the Aquaculture Performance of Cobia (Rachycentron canadum) and Yellowfin Tuna (Thunnus albacares)

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Effects of probiotics pulse-supplementation on cobia were assessed over two experimental trials carried out with larval and juvenile stages using a commercial blend of Bacillus subtilis, B. lincheniformis, B. magaterium, and Brevibacillus laterosporus. The first trial evaluated the effects of probiotic-enriched live feeds (7x10^6 and 10x10^6 colony-forming units (CFU) L^{-1} for rotifers and Artemia sp., respectively) on the growth and survival of fingerlings during a commercial larval rearing run. The second trial assessed the effects of probiotics supplementation in dry feeds (10^9 CFU kg^{-1}) on the survival, growth, feed conversion ratio (FCR), ammonia excretion of cobia juveniles (58 g to 216 g) and key metabolites measured in whole digestive systems. Results from both experiments showed no significant differences between control and probiotics groups in any of the measured variables, suggesting that pulsed-probiotic supplementation under the present conditions did not affect cobia aquaculture performance. Gas chromatography (GC) and capillary electrophoresis (CE) results evidenced similar profiles between groups for fatty acids and cations, respectively, whereas no values were detected for organic acids of low molecular weight. With regard to the free amino acid
profiles, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) assessments revealed a similar amino acid composition for both experimental groups in each pulse period. However, the concentration of each amino acid increased over time in the supplementation group, whereas the control group showed an opposite tendency. The total free amino acid concentration (ppm) at the end of the experiment was 1.5 and 2.8 times higher than in the control group, as evidenced by spectrophotometry and HPLC analysis, respectively. This increased concentration could be related to higher free amino acid availability from food hydrolysis modulated by probiotics; however, this increment had a negligible effect on feed conversion and growth.

With regards to yellowfin tuna, the effects of probiotics and 1-monoglycerides supplementation on the larval growth and survival at -10 DPH, a common age of acute mortality largely due to nutritional deficiencies, was studied. Two probiotic trials (carried out at different stocking density and different probiotic inclusion) and one trial with 1-monoglycerides were performed. The higher probiotic supplementation (40x10^6 CFU L^{-1}; Trial 2) yielded a significantly higher total length in the supplementation group with respect to the control in 10 DPH YFT larvae, with no significant differences in survival between groups for both trials. In the case of 1-monoglycerides, no significant differences were found in both variables either, although the supplementation group yielded a two-fold increase in survival compared to the control group, and overall, had higher and less variable values. Probiotics might have assisted digestive processes of the rudimentary digestive system of pre-flexion larvae, whereas 1-monoglycerides might have contributed as additional energy sources, both enabling higher larval nutrient uptake and growth.
To Nina, Diego and Laura
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CHAPTER 1: 
INTRODUCTION

OVERVIEW

Fish are one of the most traded food commodities in the world, with the aquaculture sector currently accounting for more than 50% of the fish intended for human consumption. With many commercial fisheries already reaching or surpassing their maximum yield, the aquaculture sector is expected to reach 62% by 2030 to cope with the increased demand and population growth, requiring the expansion of aquaculture operations, particularly in marine waters where there exists less conflicts of interest compared to freshwater or land (FAO, 2016, 2017; Benetti, et al., 2006, Benetti et al., 2007, Benetti and Welsh, 2010; Benetti et al., 2010b; Figure 1.1.).

Figure 1.1. World fish utilization and supply for year 2015. Taken from FAO (2017).
Among pelagic species specifically, the aquaculture potential of cobia and yellowfin tuna is of particular interest as a result of the species’ high metabolic rates, fast growth, high quality fillet and high market prices, making them ideal candidates for mariculture expansion. Cobia aquaculture developed in Taiwan, the Americas and the Caribbean in the early 2000s as a result of the species’ wide distribution, rapid growth rates, high fecundity and excellent palatability (Liao et al. 2004, Benetti, 2008, Benetti et al. 2008a, 2008b, 2010a, 2010c, Hoenig et al., 2009). Tuna aquaculture developed recently with the closed cycle production of the Pacific bluefin tuna (*Thunnus orientalis*) in 2002 in Japan after more than 40 years of research (Sawada, 2005), followed by the closed cycle production of Atlantic bluefin tuna (*Thunnus thynnus*) in Spain in 2016 (Ortega and De la Gándara, 2017) as a result of several collaborative efforts started in the early 2000s. With regards to yellowfin tuna, efforts intended for closing the production cycle of this species are showing promising results. A land-based broodstock population held since 1996 by the Inter-American Tropical Tuna Commission (IATTC) in Panama has resulted in the first generation of hatchery reared yellowfin tuna juveniles put out to sea for experimental grow-out in a cage (Benetti et al., 2016, Margulies et al., 2016).

Despite recent advances in marine fish farming (e.g. amberjacks *Seriola* spp., cobia *R. canadum*, snappers *Lutjanus* spp., pompanos *Trachinotus* spp., striped bass *Morone saxatilis*, tunas *Thunnus* spp., etc.), the rapid expansion of aquaculture operations poses both environmental and health threats due to potential eutrophication of marine areas and use of chemicals and antibiotics. Intensive carnivorous fish farming relies on dry feeds made mainly from fishmeal (30-45%) and fish oil (20%), which after catabolism, release nitrogenous wastes and feces that negatively impact the water column.
and sea bottom at different magnitudes depending on the hydrodynamics (e.g. depth and currents) and scale of the particular concession site (Benetti et al., 2006, Benetti et al., 2010b). In addition, high-density cultures increase stressful conditions where pathogen proliferation could lead to disease outbreaks, mortality, environmental deterioration and economic losses (Gatesoupe, 1999; Wang et al., 2008; Hong et al., 2005; Merrifield et al., 2010, Doulliet, 2000). Chemical additives and veterinary drugs, especially antibiotics, are customarily used for the prevention and control of animal diseases, which may promote the selection, propagation, and persistence of antibiotic-resistant strains (Martínez Cruz et al., 2012). Contrarily, probiotics, generally defined as microbial feed supplements that improve the host's intestinal balance (Fuller, 1989), have been proposed as an alternative to antibiotics and chemicals as they provide metabolites (e.g. enzymes, amino acids, and vitamins) that may boost the host's immune system and fitness. During the last decade, probiotics supplementation in several commercially reared teleost species has resulted in improvements in immune response, enzymatic activity, feed efficiency, growth, survival and ammonia excretion (Avella et al., 2012; Gatesoupe et al., 1989; Kozasa, 1986; Merrifield et al., 2010). Probiotics adhere and eventually colonize the mucosal surfaces of the gastrointestinal tract, assisting digestion and competing against potential pathogen bacteria for binding sites and nutrients, while also inducing immune-modulation (Westerdahl et al., 1991, Direkbusarakom et al., 1998, Balcázar, 2006).

In marine fish, the most widely used probiotic organisms employed to enhance growth and immune response among autochthonous (isolated from the same cultured animal) or allochthonous (from a foreign environment) bacteria species are *Bacillus* spp. and *Lactobacillus* spp. (Wang et al., 2008; Van Hai, 2015). These organisms are typically
delivered in feeds and/or rearing water in the form of live cultures, lyophilized cells, dead cells, disrupted cells, cell-free supernatants, and spores (Martínez Cruz et al., 2012), with mixtures of synergistic strains considered more effective than single strains (Van Hai, 2015; Douillet, 2000; Zink et al., 2011). Three supplementation strategies are commonly used: short-term supplementation, constant supplementation and short-term-cyclic or pulse-administration (Merrifield et al., 2010). In the case of pelagic species, in which larvae actively prey on zooplankton at early developmental stages, probiotics are added into the enrichment feed solution for live feeds (e.g., rotifers and *Artemia* sp.).

Improvements in health and growth under probiotics supplementation have been reported in salmonids (*Salmo salar, Oncorhynchus mykiss*; Merrifield et al., 2010), flounder (*Paralichthys olivaceus, Solea solea*; Nguyen et al., 2017; Palermo et al., 2011; Ye et al., 2011; Avella et al., 2011), grouper (*Epinephelus coioides*; Sun et al., 2010), seabream (*Sparus aurata*; Avella et al., 2010; Guzman-Villanueva et al., 2014) and cobia (*Rachycentron canadum*; Geng et al., 2011, 2012; He, 2015, Garrido-Pereira, 2014). Supplementation aims to maintain a high bacteria population to allow gastro-intestinal tract colonization, defined by Conway (1996) as bacterium persistence due to a multiplication rate higher than the expulsion rate. In probiotics inclusion, this multiplication rate can be modified or substituted by appropriate supplementation. However, two molecular studies carried with Atlantic cod (*Gadus morhua*) larvae have shown that supplemented bacteria might not be persistent in the digestive tract, having little effect on the larval microbiota (Skjermo et al., 2015; Bakke et al., 2013).

In the case of cobia and yellowfin tuna, despite recent advances, aquaculture performance of both species continues to be affected by low survival rates at the hatchery
level and high conversion ratios at the grow-out phase. Survival up to fully
metamorphosed cobia fingerlings, while still low compared to other species, have
improved over the last years, ranging from 5-20%. On the other hand, high mortality
rates continue to occur among tunas during the pre-flexion to flexion stage transition, at
9-10 days post hatch (DPH), with very low survival at the fingerling stage (1-3%;
Margulies et al., 2016). There are only four available references on probiotics
supplementation of cobia addressing these issues, three of them carried out with juveniles
(Geng et al., 2011, 2012; He, 2015), and one carried out with fish at early developmental
stages (Garrido-Pereira et al., 2014). There has been no previous published research on
probiotics supplementation in yellowfin tuna larvae or juveniles.

Previous work on probiotics supplementation carried by the University of Miami
Experimental Hatchery (UMEH) and the IATTC showed that the addition of probiotics
(1.5x10^9 CFU L^{-1}) to shipping bag water for yellowfin tuna larvae (simulated)
transportation resulted in significantly lower final concentrations of total ammonia
nitrogen and un-ionized ammonia and a significantly higher final mean dissolved oxygen
concentration in comparison with the control. Although no significant differences were
found in survival, the improvements in the water quality variables after a 24-hour mock
shipment suggest that the use of *Bacillus* spp. could improve safety and reduce chances
of negative results during larvae transportation (Zink et al., 2011). Additionally,
probiotics supplementation has shown improvements in the culture of the classical live
feed, rotifer *Brachionus* spp., by significantly increasing the daily mean rotifer population
on multiple culture days while reducing its variability (Zink et al., 2013). Subsequent live
feeds culture trials at UMEH showed that probiotics inclusion at 7x10^6 CFU L^{-1} for
rotifers and $10 \times 10^6$ CFU L$^{-1}$ in Artemia sp. were effective dosages for improving live feeds production in commercial hatcheries. Although these probiotic-enriched live feeds have been customarily used to feed the fish larvae as part of the larval rearing protocols, the effects of such supplementation on physiological response had not yet been investigated.

OBJECTIVES

Based on previous results from probiotics studies carried out with different marine fish species of aquaculture importance and those obtained by UMEH on enriched live feeds and yellowfin tuna larvae (simulated) transportation, this dissertation investigated the effects of probiotics supplementation on key aquaculture performance metrics in effort to improve pelagic fish production at the hatchery and grow-out farm levels where high mortality is generally caused by poor nutrition at early developmental stages and where elevated feed conversion ratios are negatively affecting the industry at grow-out operations. A series of experimental trials with larval and juvenile cobia and yellowfin tuna larvae were performed at the hatcheries of Open Blue sea farms (Caribbean side of Panama) and the Achotines Laboratory of the Inter-American Tropical Tuna Commission (IATTC) (Pacific side of Panama), respectively, to assess the effects of probiotics supplementation on:

i) growth and survival of cobia fingerlings and yellowfin tuna larvae at 9-10 DPH; and

ii) growth, survival, feed conversion ratio and ammonia excretion and
key metabolites profile (amino acids, fatty acids, cations and organic acids of low molecular weight) on the digestive systems of juvenile cobia.

One additional experiment was also conducted using 1-monglycerides supplementation to assess yellowfin tuna survival and growth at early development, as these compounds have been reported to also modify bacterial communities in the fish gut with positive impact on survival and growth in other aquatic species (Parini, 2016). Much of the information currently available on probiotics and aquaculture is based on average performance species (Avella et al., 2012; Gatesoupe et al., 1989; Kozasa, 1986; Merrifield et al., 2010, Nguyen et al., 2017; Palermo et al., 2011; Ye et al., 2011; Avella et al., 2011, Sun et al., 2010, Avella et al., 2010; Guzman-Villanueva et al., 2014), with the results of this study representing one of the few available attempts to describe how probiotics may influence the performance of larvae and juveniles of two commercially important, high-performance pelagic species under controlled culture conditions. Specifically, the following questions were addressed:

A) Does probiotics supplementation customarily used for improving live feeds production significantly improve growth and survival in cobia fingerlings and YFT larvae at 9-10 DPH?

B) Does probiotics-supplemented dry feed significantly affect growth, feed conversion efficiency, ammonia excretion and survival in juvenile cobias?

C) Can the supplemented microbiota modify the fatty acid, amino acid, cations and organic acids of low molecular weight in digestive tract systems of juvenile cobia?
D) Does the supplementation of rotifers with 1-monoglycerides affect the growth and survival of YFT larvae at 9-10 DPH?

BACKGROUND AND SIGNIFICANCE

Global projections estimate significant challenges in supplying the rapidly growing human population with limited resources. Water is a critical resource for food and energy production, as well as manufacturing, with 20% of the world’s aquifers currently over-exploited. The estimates for 2050 are even more concerning. To cope with the requirements of an expected population of 9.6 billion, demand for water is expected to increase by 55%, food by 60%, energy consumption by 25-50% and aquaculture by more than 100% (Alexandratos and Bruinsma, 2012; World Energy Council, 2013, FAO, 2010; WWAP, 2015; Waite et al., 2014). In the context of climate change and its effects on hydrological regimens, this food security scenario demands a shift in the way society develops production systems and use natural resources.

While fish aquaculture is less dependent on feed supplies than any other form of animal farming among vertebrate species (MarineHarvest, 2017; Ytrest_yl et al., 2014, 2015; Jackson, 2009; Volden and Nielsen, 2011; Winther Ulf and Harald, 2009; Mekonnen and Hoekstra, 2010; Welsh et al., 2010; Figure 1.2), the aquaculture industry must develop technologies for producing more fish while minimizing its reliance on land, freshwater, feed and energy. Given that low survival rates at the larval stages and high feed conversion ratios at grow-out phase represent the main constraints for the pelagics aquaculture industry, this study addresses both issues from a nutritional perspective, evaluating the effect of feed supplements, particularly probiotics and 1-monoglycerides,
on the aquaculture performance of cobia at larval and juvenile stages and YFT at the early developmental stage.

**Figure 1.2.** Production efficiency, carbon footprint and water consumption among the animal farming industry (Taken from Marine Harvest, 2015).
CHAPTER 2:

PROBIOTICS EFFECTS ON GROWTH, SURVIVAL, FEED CONVERSION EFFICIENCY AND AMMONIA EXCRETION

SUMMARY

Effects of probiotics pulse-supplementation on cobia were assessed over two experimental trials carried with larval and juvenile stages using a commercial blend of *Bacillus subtilis*, *B. lincheniformis*, *B. magaterium*, and *Brevibacillus laterosporus*. The first trial evaluated the effects of probiotic-enriched live feeds (7x10^6 and 10x10^6 colony-forming units (CFU) L^-1 for rotifers and *Artemia* sp., respectively) on the growth and survival of fingerlings during a commercial larval rearing run; the second trial assessed the effects of probiotics supplementation in dry feeds (10^9 CFU kg^-1) on the survival, growth, feed conversion ratio (FCR) and ammonia excretion of cobia juveniles (58 g to 216 g). Results from both experiments showed no significant differences between control and probiotics groups in any of the measured variables, suggesting that pulsed-probiotic supplementation under the present conditions did not affect cobia aquaculture performance.

BACKGROUND

Regarding probiotic supplementation in cobia (*Rachycentron canadum*), three studies in juveniles described improved digestion, growth, immune response and survival (Geng et al., 2011, 2012; He, 2015), whereas one study with larvae in recirculating aquaculture system (RAS) did not show differences in growth and water quality between control and
probiotics-treated group (Garrido-Pereira et al., 2014). In the western hemisphere, cobia is commercially reared in Panama and Ecuador and was developed from collaborations between the private sector and the University of Miami Experimental Hatchery (UMEH) on site-selection, broodstock/larval rearing and grow-out operations. In this work, two experimental trials were performed at the commercial hatchery of Open Blue Sea Farms (Caribbean coast of Panama). The first trial tested whether the probiotic dosages customarily used in the live feeds production ($7 \times 10^6$ CFU L$^{-1}$ for rotifers and $10 \times 10^6$ CFU L$^{-1}$ for *Artemia* sp.) had any effect on the growth and survival from first feeding larvae to complete metamorphosis fingerlings during a commercial larval rearing run. The second trial tested probiotic inclusion in dry feeds ($10^9$ CFU kg$^{-1}$) for juvenile production regarding growth, survival, FCR and ammonia excretion.

MATERIALS AND METHODS

**Probiotics source and supplementation strategies.** A liquid bacterial blend of *Bacillus subtilis*, *B. lincheniformis*, *B. megaterium*, and *Brevibacillus laterosporus* ($1 \times 10^8$ CFU mL$^{-1}$; Ecomicrobials, LLC) was delivered in both experimental trials through a short-term-cyclic (pulse)-administration consisting of three periods: 15 days of probiotic supplementation, a withdrawal period of seven days (probiotic group was fed the same live feeds as the control group), and an additional probiotic supplementation of 7 days.

**Larval developmental stages - from first feeding larvae to 1 g fingerlings.** Live feeds rooms were equipped with both, control and probiotics enrichment units. Rotifers were enriched for three consecutive days with 0.2-0.6 g Orione® (Skretting, Norway) day$^{-1}$ for every $10^6$ rotifers; probiotic-treated enrichment units were additionally
supplemented with 7x10^6 CFU L⁻¹. *Artemia* sp. was enriched overnight for 12 hours with a mix of commercial products containing Algamac (75%; Aquafauna Biomarine Inc., Hawthorne, CA, USA), Astaxanthin (15%; Astarose; Aquafauna Biomarine Inc.), Protein Plus (10%; Aquafauna Biomarine Inc.) and 100 mL of algae paste (*Nannochloropsis* sp.; Nanno 3600, Reed Mariculture, Campbell, CA, USA) using 0.3 g of this mix for every 10^6 *Artemia* sp., adding 10x10^6 CFU L⁻¹ in the probiotics-supplemented units. Larvae and fingerlings were fed seven times a day over the duration of the study, delivering rotifers from 3 to 12 days post-hatch (DPH), *Artemia* sp. from 8-28 DPH and dry feeds (Otohime B1, B2, C1, C2 and EP1) from 15 DPH on, increasing the pellet size according to fish development (Table 2.1).

**Table 2.1.** Cobia feeding schedule and daily rations of live and dry feeds used in the study at early developmental stages

<table>
<thead>
<tr>
<th>DPH</th>
<th>Total Live Feeds mL⁻¹ day⁻¹</th>
<th>Dry Feeds</th>
<th>Time</th>
<th>Feed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10</td>
<td>0.3</td>
<td>6:00</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>0.5</td>
<td>8:00</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>1.0</td>
<td>10:00</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>1.3</td>
<td>12:00</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>3.0</td>
<td>14:00</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>3.3</td>
<td>16:00</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>4.0</td>
<td>17:00</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>4.0</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>B2/B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>B1/B2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>B2/C1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>B2/C1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>B1/B2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>B1/B2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>B2/C1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>B2/C1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>2.5</td>
<td>C1/C2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22-34</td>
<td>2.5</td>
<td>C1/C2/EP1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Juvenile stage - dry feeds enrichment and feeding schedule.** Dry feed Nicovita Classic Cobia® (4 and 8 mm; Alicorp, Peru) was supplemented with $10^9$ CFU kg$^{-1}$ using flavorless jelly as a binding agent, whereas dry feeds for the control group were coated with the flavorless jelly only. Feeds were prepared daily in an isolated room and allowed to dry overnight at 18°C. Fish were fed to apparent satiation twice a day (8:00 and 14:00 hours), with the amount of consumed feed registered for each rearing unit; feeding was stopped once feeding activity reduced and the first uneaten pellets sank to the bottom.

**Experimental setup and variables - Larval developmental stages.** Eight tanks of 10,000 L each were stocked with 680,000 2 DPH cobia larvae (8.5 larvae L$^{-1}$) coming from the same volitionally spawn (730,000 eggs; 83% of fertilization ratio; 98% of hatching ratio). Two treatments were tested, control and probiotics supplementation, each consisting of four replicates. In each rearing unit, 60 fish were sampled at the end of each pulsed-supplementation period (18, 24 and 34 DPH) euthanized using 40 ppm Eugenol. Total length (mm) and weight (g) were measured using a digital caliper and a digital scale, respectively. Survival (final number of fish produced) was estimated at the end of the larval rearing stage (34 DPH) in each experimental unit, using an automated fish counter (Vaki Macro EXEL) adapted to a fish pump (Vaki Heathro Fish Pump 6), also used to transfer the fish from the larval rearing units into two nursery tanks of 100,000 L, keeping the groups (control and probiotic) separated for further growth monitoring on 44, 47 and 53 DPH.

**Experimental setup and variables - Juvenile stage.** Fish used in this experiment came from the nursery tank control population described in the previous section. At 68 DPH, 2,520 cobia juveniles of uniform size (58.39 ± 1.56 g of body weight) were
collected and equally distributed into eight 8,000 L tanks (n=315 fish/tank). Two groups, control and probiotics, were evaluated, each with four replicates randomly assigned. The following variables were calculated: 1) Specific growth rate (SGR) = (Ln Wt - Ln W0) x 100/t, where Wt and W0 were final and initial weight of cobia, respectively, and t total period in days. 2) Survival = Amount of total fish produced in each unit at the end of the experiment. 3) Feed conversion ratio (FCR) = kg of feed consumed / kg of fish produced and 4) Ammonia excretion (ppm) = Total ammonia nitrogen (TAN; mg NH₃N and NH₄ L⁻¹). Fifty fish in each of the experimental units were sampled for weight assessments once a week, with the exception of the last sampling in which 100 fish in each tank were measured. Fish were anesthetized with eugenol solution (20 ppm), individually weighted, and transferred back to the tank. Feed consumption was registered daily to estimate the feed conversion ratio at each sampling period and at the end of the experiment based on the biomass gain. Ammonia excretion along the experiment was measured two hours post-feeding, either in the morning, afternoon or both using an electronic La Motte Smart 3 colorimeter kit for marine water. Survival (final number of fish produced in each experimental units) was calculated at the end of the experiment by counting the fish individually.

**Statistical Analysis.** Data sets from each variable were checked for outliers, normality and homoscedasticity and tested for differences between groups with parametric analyses using the statistic software SPSS 24®(IBM®) with a significance level of α = 0.05. Data are mean ± standard deviation, unless otherwise stated.

**Early developmental stages.** Difference in growth between experimental groups was tested with a one-way ANCOVA using natural logarithm-transformed data,
considering LN Length (mm) as the dependent variable and LN Weight (g) as the covariate. Survival (final number of fish produced) was assessed through an independent-samples t-test.

**Juvenile stage.** A one-way ANCOVA was run to test for significant differences in growth between groups. Untransformed weight (g) values were considered as the dependent variable and DPH as the covariate, as a result of the linear trend displayed by the portion of the growth curve obtained in this trial (68 to 96 DPH; Figure 2.1). An independent t-test was run to test for differences in survival and ammonia excretion.

![Figure 2.1](image.png)

**Figure 2.1.** Cobia growth curve. Left, overall growth of the cobra cohort from 36 to 96 DPH. Right, growth of juvenile cobra for the particular experimentation period of 28 days (68-96 DPH)

RESULTS

**Cobia - early stages of development – Growth.** There was a linear relationship between LN Length (mm) and LN Weight (g) for each experimental group (Figure 2.2), homogeneity of regression slopes as the interaction term was not statistically significant (F (1, 20) = 0.03; p = 0.864) and homogeneity of variances (Levene's test p = 0.171).
After adjustment for weight, growth was slightly higher in the control group (3.307 ± 0.037) compared to the probiotics group (3.249 ± 0.037; Table 2.2), with no statistically significant difference between them F(1, 21)=1.267, p=0.273, partial η² = 0.057 (Table 2.3).

Figure 2.2. Cobia at early stages of development. Growth regression lines and equations for control group and probiotics group.

Table 2.2. Adjusted and unadjusted means and variability for LN Length (mm) with LN Weight as a covariate for cobia at early stages of development. N= number of observations, M=Mean, SD=Standard deviation, SE=Standard error. Length measured in mm; LN transformations were applied to the ANCOVA analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Unadjusted M</th>
<th>SD</th>
<th>Adjusted M</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>3.270</td>
<td>0.738</td>
<td>3.307</td>
<td>0.037</td>
</tr>
<tr>
<td>Probiotics</td>
<td>12</td>
<td>3.287</td>
<td>0.720</td>
<td>3.249</td>
<td>0.037</td>
</tr>
</tbody>
</table>
Table 2.3. ANCOVA output for cobia growth (LN Length) at early stages of development between experimental groups (test of between-subjects effects)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
<th>Partial η Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected model</td>
<td>11.366²</td>
<td>2</td>
<td>5.683</td>
<td>354.759</td>
<td>.000</td>
<td>.971</td>
</tr>
<tr>
<td>Intercept</td>
<td>165.357</td>
<td>1</td>
<td>165.357</td>
<td>10322.519</td>
<td>.000</td>
<td>.998</td>
</tr>
<tr>
<td>LN W (g)</td>
<td>11.364</td>
<td>1</td>
<td>11.364</td>
<td>709.411</td>
<td>.000</td>
<td>.971</td>
</tr>
<tr>
<td>Group</td>
<td>.020</td>
<td>1</td>
<td>.020</td>
<td>1.267</td>
<td>.273</td>
<td>.057</td>
</tr>
<tr>
<td>Error</td>
<td>.336</td>
<td>21</td>
<td>.016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>269.633</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>11,702</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. R Squared = .971 (Adjusted R Squared = .969)

Cobia - early stages of development – Survival. The control group had higher survival and less variability than the probiotics group (Table 2.4), with no significant differences detected in the number of fish produced between control group and probiotics group by the independent-samples t-test (mean difference = 598.75 fingerlings; 95% CI, -783.7 to 1973.2 not statistically significant, t(6) = 1056, p = 0.332; Table 2.5; Figure 2.3).

Table 2.4. Group statistics for final number of fish produced between control and probiotics groups in the cobia early stages of development trial

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>S.D.</th>
<th>S.E. Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>4894.50</td>
<td>85.676</td>
<td>42.838</td>
</tr>
<tr>
<td>Probiotics</td>
<td>4</td>
<td>4299.75</td>
<td>1123.464</td>
<td>561.732</td>
</tr>
</tbody>
</table>

Table 2.5. Independent samples t-test for the final number of fish produced in both treatments at the end of the cobia early stages of development trial

| Equal variances assumed | 4,792 | .071 | 1,056 | .332 | 594,750 | 563,303 | -783,749 | 1973,249 |
| Equal variances not assumed | 1,056 | .968 | 3,035 | .368 | 594,750 | 563,303 | -1186,517 | 2376,017 |
Figure 2.3. Boxplot comparing the final number of fish produced between control and probiotics groups at the end of the cobia early stages of development - Fingerlings trial

Cobia Juvenile stage – Growth. There was a linear relationship between weight (g) and DPH (Figure 2.4) with homogeneity of regression slopes as the interaction term was not statistically significant (F (1, 36) = 0.020; p = 0.888) and homogeneity of variances (Levene's p = 0.377). Weight was slightly higher in the probiotics group compared to the control group with no statistically significant difference between them (F (1,37) = 1.507, p=0.227, partial η2 = 0.39; Tables 2.6 and 2.7). After 28 days under probiotic pulse-supplementation, juveniles grew at a rate of 5.7 g day⁻¹, from 58.75 ± 8.3 g to 218.57 ± 33.21 g in control group and from 58.03 ± 8.2 to 216.29 ± 32.97 g in the probiotics group, both exhibiting a specific growth rate (SGR) of 4.7%.

The few available references on other probiotics experiments performed with juvenile cobia involve fish of different size and different rearing periods than the present work. For comparison purposes, estimations of age and weight for the control fish cohort used in this study, depicted from the general growth curve equation described in Figure
2.1. (Left), were made for matching the weight ranges portrayed by Geng et al. (2011) and Geng et al. (2012). Likewise, estimations of final weight derived from the SGR provided by those authors were also made for contrasting growth rates (Table 2.9). Geng et al. (2011) obtained a SGR ranging from 1.99% to 2.57% day$^{-1}$, depending on the supplementation type after a rearing period of 56 days. Based on those SGR, it was estimated that juveniles grew from 10.1 g to a weight range of 111.44 g to 143.92 g (mean for all treatments =128.8 g; $\approx$ 2 g day$^{-1}$).

![Graph showing growth regression lines and equations for both groups.]

**Figure 2.4.** Cobia growth at juvenile stage. Left, conventional growth curve for cobia; period evaluated in this trial is highlighted in a box; right, scatterplot of the untransformed weight data and DPH, showing growth regression lines and equations for both groups.

The growth of the fish cohort used in the present juvenile study was recorded at four intermediate ages (33, 44, 47 and 53 DPH) between the end of the larval rearing trial (34 DPH) and the start of the juvenile fish experiment (68 DPH). At 47 DPH, juveniles in
the present work had a similar size (10.00 g) of that displayed by the fish at the beginning of the experiment done by Geng et al. (2011). Based on the exponential equation for control fish population showed in Figure 1A ($Y = 2 \times 10^{-8} \times 5.18$), it was estimated that fish in the present study reached the mean final weight of 122.8 g obtained by Geng et al. (2011) at 77.5 DPH, equivalent to a growth period of 30.5 days. The estimated SGR for this control fish cohort from 10 g to $\approx 122.8$ g during the 30.5 days period (47 DPH to 75.5 DPH) was 8.22% day$^{-1}$, indicating that fish in the present study grew 3.7 times faster in about half the time (54% less time) in comparison to the mean SGR results reported by Geng et al. (2011), in which the SGR was 1.99% to 2.57% day$^{-1}$ (mean SGR = 2.2% day$^{-1}$) in a 56 days period. The same approach was followed for comparing the growth rates obtained in the present trial with a posterior study made by Geng et al. (2012), estimating the SGR for the weight interval of 9.4 g to 46.4 g (initial and average final weight for all treatments obtained by Geng et al. (2012)). The estimated age for the fish in the present study for matching the initial weight of 9.4 g was 47 DPH whereas the estimated age for matching the final weight of 46.4 g was 64 DPH, that is to say a growth period of 17.2 days. The corresponding estimated SGR for the fish in the present study during this 17.2 days period was 9.3% day$^{-1}$, indicating that fish of the present study grew between 3.4 to 2.5 times faster in about 30% of the time compared with that obtained by Geng et al. (2012), in which the SGR ranged between 2.71 to 3.74% day$^{-1}$.

**Cobia Juvenile stage - Survival.** A total of 2,118 juvenile cobia were counted between both groups, with control and probiotics treatment accounting for 50.4% and 49.6% of the fish, respectively (Table 2.10). The mean number of fish produced was
slightly higher in control group (267.01 ± 17.86) than in probiotics group (255.64 ±19.39; Figure 5; Table 2.11), with the mean difference of 11.37 (95% CI, -20.88 to 43.62) from the independent samples t-test being not statistically significant, t(6) = 0.863, p = 0.421 (Table 2.12).

**Table 2.6.** Growth of cobia juveniles during probiotic pulse-supplementation for 28 days

<table>
<thead>
<tr>
<th>DPH</th>
<th>Mean weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>68</td>
<td>58.7 ± 8.3</td>
</tr>
<tr>
<td>75</td>
<td>95.3 ± 13.5</td>
</tr>
<tr>
<td>81</td>
<td>141.6 ± 25.4</td>
</tr>
<tr>
<td>89</td>
<td>171.5 ± 26.9</td>
</tr>
<tr>
<td>96</td>
<td>218.6 ± 33.2</td>
</tr>
</tbody>
</table>

**Table 2.7.** Adjusted and unadjusted means and variability for Weight (g) with DPH as a covariate for cobia at juvenile stage. N= number of observations, M=Mean, SD=Standard deviation, SE=Standard error. Weight measured in g

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Unadjusted M</th>
<th>Unadjusted SD</th>
<th>Adjusted M</th>
<th>Adjusted SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>133.82</td>
<td>57.34</td>
<td>133.82</td>
<td>1.91</td>
</tr>
<tr>
<td>Probiotics</td>
<td>20</td>
<td>137.14</td>
<td>58.070</td>
<td>137.44</td>
<td>1.91</td>
</tr>
</tbody>
</table>

**Cobia Juvenile stage – Feed conversion ratio (FCR).** Total given feed, increment in fish biomass and hence FCR were similar among all experimental units (Table 11). On average, control tanks had a FCR of 1.14 whereas probiotic tanks had a FCR of 1.16 (Figure 6). An independent-samples t-test on the overall FCR showed that the mean difference of -0.027 (95% CI, -0.241 to 0.186) was not statistically significant, t(6) = 0.315, p = 0.763 (Table 12).
Table 2.8. ANCOVA output for cobia juveniles growth (Weight g) between experimental groups (test of between-subjects effects)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
<th>Partial $\eta^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected model</td>
<td>123950,040</td>
<td>2</td>
<td>61975,020</td>
<td>846,420</td>
<td>.000</td>
<td>.979</td>
</tr>
<tr>
<td>Intercept</td>
<td>60675,914</td>
<td>1</td>
<td>60675,914</td>
<td>828,678</td>
<td>.000</td>
<td>.957</td>
</tr>
<tr>
<td>DPH</td>
<td>123839,683</td>
<td>1</td>
<td>123839,683</td>
<td>1691,333</td>
<td>.000</td>
<td>.979</td>
</tr>
<tr>
<td>Group</td>
<td>110,357</td>
<td>1</td>
<td>110,357</td>
<td>1,507</td>
<td>.227</td>
<td>.039</td>
</tr>
<tr>
<td>Error</td>
<td>2709,146</td>
<td>37</td>
<td>73,220</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>860884,917</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>126659,186</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. R Squared = .979 (Adjusted R Squared = .977)

Figure 2.5. Boxplot comparing the final number of fish produced between control and probiotics groups at the end of the juvenile stage trial.

Table 2.9. Comparison of juvenile cobia growth between different experiments

<table>
<thead>
<tr>
<th>Reference</th>
<th>Rearing period</th>
<th>Initial W</th>
<th>Final Weight</th>
<th>SGR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(days)</td>
<td>(g)</td>
<td>(g)</td>
<td>(%)</td>
</tr>
<tr>
<td>Present work</td>
<td>28</td>
<td>58.7</td>
<td>218.6</td>
<td>4.70</td>
</tr>
<tr>
<td>Geng et al. (2011)</td>
<td>56</td>
<td>10.1</td>
<td>114.4 - 143.9</td>
<td>1.99 - 2.57</td>
</tr>
<tr>
<td>Present work**</td>
<td>30.5</td>
<td>10.0</td>
<td>128.8</td>
<td>8.22</td>
</tr>
<tr>
<td>Geng et al. (2012)</td>
<td>56</td>
<td>9.4</td>
<td>46.4</td>
<td>2.71 - 3.74</td>
</tr>
<tr>
<td>Present work**</td>
<td>17.2</td>
<td>9.4</td>
<td>46.4</td>
<td>9.3</td>
</tr>
</tbody>
</table>
Table 2.10. Biomass, total fish produced and survival for both groups

<table>
<thead>
<tr>
<th>Group / Yield</th>
<th>Tank</th>
<th>Biomass (Kg)</th>
<th>Fish left</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 231 Kg</td>
<td>13</td>
<td>62</td>
<td>287</td>
<td>91.0</td>
</tr>
<tr>
<td>1,068 fish</td>
<td>16</td>
<td>60</td>
<td>277</td>
<td>88.1</td>
</tr>
<tr>
<td>Probiotics</td>
<td>14</td>
<td>53</td>
<td>238</td>
<td>75.5</td>
</tr>
<tr>
<td>229.5 Kg</td>
<td>15</td>
<td>58</td>
<td>265</td>
<td>84.2</td>
</tr>
<tr>
<td>1,050 fish</td>
<td>18</td>
<td>53</td>
<td>241</td>
<td>76.5</td>
</tr>
<tr>
<td>Control 19</td>
<td>19</td>
<td>60</td>
<td>278</td>
<td>88.3</td>
</tr>
</tbody>
</table>

Table 2.11. Group statistics for final number of fish produced between control and probiotics groups in the cobia juvenile stage trial.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>S.D.</th>
<th>S.E. Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>4894.50</td>
<td>85.67</td>
<td>42.838</td>
</tr>
<tr>
<td>Probiotics</td>
<td>4</td>
<td>4299.75</td>
<td>1123.46</td>
<td>561.732</td>
</tr>
</tbody>
</table>

Table 2.12. Independent samples t-test for the final number of fish produced in both treatments at the end of the cobia juvenile stage trial.

<table>
<thead>
<tr>
<th></th>
<th>Levene's Test</th>
<th>t</th>
<th>df</th>
<th>Sig.</th>
<th>t-test</th>
<th>95% C.I. of the Diff.</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>Sig.</td>
<td>t</td>
<td>df</td>
<td>Sig.</td>
<td>Mean Diff.</td>
<td>S.E. Diff.</td>
<td></td>
</tr>
<tr>
<td>Equal variances assumed</td>
<td>,103</td>
<td>.759</td>
<td>,863</td>
<td>6</td>
<td>,421</td>
<td>11,370</td>
<td>13,179</td>
<td>-20,878</td>
</tr>
<tr>
<td>Equal variances not assumed</td>
<td>5,960</td>
<td>,422</td>
<td>6</td>
<td>,421</td>
<td>11,370</td>
<td>13,179</td>
<td>-20,930</td>
<td>43,670</td>
</tr>
</tbody>
</table>

Table 2.13. Total food consumed, fish biomass gain and FCR at the end of the cobia juvenile stage trial in each of the experimental units.

<table>
<thead>
<tr>
<th>Control tank No.</th>
<th>Probiotic tank No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feed (Kg)</td>
</tr>
<tr>
<td>13</td>
<td>45.888</td>
</tr>
<tr>
<td>16</td>
<td>44.854</td>
</tr>
<tr>
<td>17</td>
<td>45.705</td>
</tr>
<tr>
<td>20</td>
<td>43.057</td>
</tr>
<tr>
<td></td>
<td>41.660</td>
</tr>
<tr>
<td></td>
<td>45.081</td>
</tr>
<tr>
<td></td>
<td>44.304</td>
</tr>
<tr>
<td></td>
<td>47.333</td>
</tr>
</tbody>
</table>
Table 2.14. Independent samples t-test for FCR at the end of the cobia juvenile trial.

<table>
<thead>
<tr>
<th>Levene's Test</th>
<th>t</th>
<th>df</th>
<th>Sig.</th>
<th>Mean Diff.</th>
<th>S.E. Diff.</th>
<th>95% C.I. of the Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equal variances assumed</td>
<td>.990</td>
<td>.555</td>
<td>.315</td>
<td>6</td>
<td>.763</td>
<td>-.02750</td>
</tr>
<tr>
<td>Equal variances not assumed</td>
<td>-.315</td>
<td>5.550</td>
<td>.764</td>
<td>6</td>
<td>.02750</td>
<td>.8726</td>
</tr>
</tbody>
</table>

Figure 2.6. Mean FCR for control and probiotics groups at the end of the cobia juvenile trial.

*Cobia Juvenile stage – Ammonia excretion.* Twelve ammonia measurements were done during the cobia juvenile trial, comparing control and probiotics group. In all cases, independent samples t-tests showed no statistically significant differences between the groups in ammonia excretion (Table 2.15).

Table 2.15. Total ammonia nitrogen concentration-TAN (mg NH₃ and NH₄ L⁻¹) in experimental tanks over the cobia juvenile trial.
DISCUSSION

Probiotics supplementation has previously demonstrated enhanced performance in several teleost species and is perceived as a biological alternative to conventional veterinary products for aquaculture. Pelagic fish farming is mostly challenged by low survival rates at the larval stage and by high FCR at grow-out operations. These conundrums are mostly addressed through nutritional approaches, complemented by other research in health and genetics. The present work tested the effects of probiotic-supplemented feeds on the growth, survival, feed conversion and ammonia excretion of cobia. One study at early developmental stages was carried out during a commercial larval rearing run testing the probiotics inclusion in live feeds on the growth and survival. A total of 54,317 cobia fingerlings (1.21 ± 0.15 g; 34 DPH) were produced between both treatments with no statistically significant differences in growth and survival between control and probiotics group, suggesting that probiotics supplementation customarily used in the production of live feeds does not affect fingerlings performance. Similar findings were reported by Garrido-Pereira et al. (2014) in a cobia larval rearing experiment on probiotics supplementation in a recirculating aquaculture systems (RAS). While probiotic-treated larvae displayed a greater resistance to salinity stress and immune system differentiation compared to the control, their results showed that survival, final weight and water quality (total ammonia-nitrogen, nitrite and nitrate) were not affected by probiotics supplementation, which was about three orders of magnitude higher than those used in this study. Garrido-Pereira et al. (2014) provided a daily addition of probiotics at 5x10^7 CFU L^{-1} for culture water and at 5x10^9 CFU L^{-1} for live feeds enrichment, whereas the present study used 7 to 10 x10^6 CFU L^{-1}. The lack of significant
differences (growth, water quality and survival) displayed in these two larval rearing experiments under such a wide supplementation range could be explained by the potential low colonization rates in the fish gut by the probiotics blends. Skjermo et al. (2015) measured the relative amounts of added bacterial strains at different developmental stages for 10 days after exposure in Atlantic cod larvae, finding that in most of cases, the added strains (Microbacterium sp., Ruegeira sp., Pseudomonas sp. and Vibrio sp.) declined to background levels after a maximum of four days, which suggests only a temporary presence in the larvae, despite of being autochthonous bacterial strains (isolated and cultured from reared cod larvae) with proven probiotic properties. Each of these bacterial strains were added in equal numbers to the fish rearing water at a concentration of $5 \times 10^9$ CFU L$^{-1}$, whereas live feeds were supplemented with an mixture of the four strains at $4 \times 10^{11}$ CFU L$^{-1}$ for 24 hours. Larvae were treated at seven different ages (0, 2, 4, 8, 16, 30 and 45 DPH) with one new experimental group started each time. A real time PCR was used to measure the relative amounts of the bacterial strains in live feeds, water and cod larvae during 10 days after exposure. Results showed that probiotic bacteria were only transiently detected in the larvae and that none of them were able to establish as persistent larval microbiota at any stage of development, which support a previous study with the same fish species, concluding that live feed microbiota had little effect on the larval microbiota (Bakke et al., 2013). Skjermo et al. (2015) suggested that manipulating the microbiota composition by introducing other strains is difficult to achieve and that a possible explanation is the rapid development displayed by the fish during the larval stages, characterized by a continuously changing selection pressure for the bacteria in the digestive tract and that for introducing probiotic bacteria to cod larvae, a continuous and
labor-intensive probiotics supply is required. Additionally, marine teleosts have evolved in a hypo-regulation strategy for handling the composition of their extracellular fluid. Since their bodies are hypotonic to their saltwater environment, they constantly lose water osmotically across the gills and must drink seawater to maintain osmotic balance through absorption of the salts and water across the gastrointestinal tract, with passive transfer of Na\(^+\) and active transport of Cl\(^-\) (Marshall and Grosell, 2006). This constant flow of water through the digestive tract might also favor the transient presence of bacteria and other microorganisms in the intestine (Martínez-Cruz et al., 2012), affecting probiotic bacteria ratios.

In relation to cobia at juvenile stage (68-96 DPH; ≈ 59-136 g), a common probiotic inclusion level in dry feeds (109 CFU kg\(^{-1}\)) was tested, obtaining no statistically significantly differences in growth, FCR, survival and ammonia excretion between control and probiotics groups after one month of pulse supplementation.

Three previous studies on probiotics supplementation carried out with cobia juveniles (≈10-133 g) addressed some of the variables evaluated in the present study (growth, survival and FCR) while also considering other variables related with immune and digestive responses. Results from these references coincide with those obtained in the present study regarding no significant differences in terms of survival, but evidenced significant differences in growth and FCR. Geng et al. (2011) combined the immunostimulant chitosan and the probiotic *Bacillus subtilis* at different levels of inclusion (3.0 and 6.0 g Kg\(^{-1}\) of chitosan mixed with 0.0, 2.0\times10^{10} \text{ and } 4.0\times10^{10} \text{ CFU } B. \textit{subtilis} \text{ Kg}^{-1} \text{ diet}) at continuous supplementation (56 days) for cobia juveniles (10.1 to 121.5-154.02 g). Most of supplemented diets yielded significantly higher growth (SGR)
and increased non-specific immune response and disease resistance, suggesting the combination of $2.0 \times 10^{10}$ CFU $B. \ subtilis$ and 6.0 g chitosan Kg$^{-1}$ feed as optimal inclusion. In a subsequent 56 day-experiment, Geng et al. (2012) obtained similar results on cobia juveniles (9.4 g to 34.9 - 52.7 g) using a probiotic blend ($Bacillus \ subtilis$ at $7.0 \times 10^{9}$ CFU g$^{-1}$; $B. \ lincheniformis$ at $3.0 \times 10^{9}$ CFU g$^{-1}$; $Lactobacillus$ sp. at $5.0 \times 10^{8}$ CFU g$^{-1}$ and $Arthrobacter$ sp. at $1.0 \times 10^{8}$ CFU g$^{-1}$) given at six different levels of inclusion (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 g Kg$^{-1}$ dry feeds); an inclusion of 3.3 g Kg$^{-1}$, determined by second-order polynomial regression analysis, was proposed as the optimal dose for improving growth and immune resistance. The third work in juveniles was performed by He et al. (2015) and yielded similar output as previous studies from Geng et al. (2011, 2012). In this third work, $Bacillus \ subtilis$ and $Lactobacillus \ acidophilus$ were delivered either mixed or separately; either at continuous feeding or at pulse supplementation, to assess its effects on growth, digestive enzymes and immune enzyme activity. Results evidenced that probiotic supplementation as separate or combined can promote the growth and boost the immune and digestive enzymes of juvenile cobia; continuous supplementation however, yielded better results than non-continuous supplementation.

One control group and six probiotic-treated groups were assessed. Control fish (T 0) were fed a basal diet for 50 days. Three different dosages were evaluated in the probiotics-treated groups: $B. \ subtilis$ at $1 \times 10^{7}$ CFU g$^{-1}$, $L. \ acidophilus$ at $1 \times 10^{7}$ CFU g$^{-1}$ and the mix of $B. \ subtilis$ and $L. \ acidophilus$ (ratio 1:1) at $2 \times 10^{7}$ CFU g$^{-1}$. These dosages were added to the feed in three groups (T1, T2 and T3, respectively) during 30 days and then withdrawn for 20 additional days, in which these groups were fed the same basal diets as the control group (T0). The other three groups (T4, T5 and T6) were fed the described
dosages during the whole period of 50 days. Supplementation levels employed in the aforementioned references were higher compared with those obtained in the present work where no significant differences in growth were found. However, estimates from the growth rate obtained here appear to be greater than those signiﬁcantly higher reported by Geng et al. (2011, 2012) at different juvenile size range. It was estimated that the control fish cohort used in the present study reached the size range described in the 2011 and 2012 references in 40% and 30% less time, respectively. These differences in growth rate between western and eastern hemisphere juvenile cobia populations can be explained by differences in genetics, nutrition and overall rearing methods, since some of the water quality variables (temperature, salinity and dissolved oxygen) were similar, whereas the final fish density was exceeded by far in the present work using 8m3 tanks (mean density = 7.1 kg 1,000 L−1), compared to those obtained by Geng et al. in 2011 (0.57 to 0.75 kg 1,000 L−1) and in 2012 (0.2 to 0.3 kg 1,000 L−1) in floating cages.

Regarding water quality, evidence supporting probiotics supplementation has been described in several fish species. Padmavathi et al. (2012) showed that probiotics supplementation (Nitrosomas sp at 1.62 Kg ha−1; Nitrobacter sp at 0.82 Kg ha−1) in earthen ponds used for Pangasius sutchi, Catla catla and Labeo rohita poly-culture reduced pathogenic bacteria and lowered the ammonia, nitrite and phosphates concentration. Raparia and Bhatnagar (2016) found that inclusion of Bacillus coagulans at 3x10^6 CFU Kg−1 feed lowered excretion and increased growth in major carp Catla catla. In tilapia Oreochromis niloticus, AbdelAzeem et al. (2001) obtained a significant reduction in ammonia, nitrate and nitrite concentration using a supplementation of Saccharomyces cerevisiae (200 g ton−1 of feed). In rainbow trout fry, Sgarzi et al. (2015)
obtained a significant decrease in ammonia excretion using a supplementation dosage of $1 \times 10^8$ CFU Kg$^{-1}$ feed. In the present work, however, no differences in ammonia excretion by Juvenile cobia were detected between control group and probiotics-supplemented group, results which concur with the ammonia excretion results provided by Garrido-Pereira et al. (2014) in cobia at early developmental stages.

CONCLUSION

Findings at the early developmental stages or at the juvenile phase suggest that pulsed-probiotic inclusion does not influence cobia aquaculture performance under the described conditions. In concurrence with results from the available references on the species, this study noted no significant difference in growth at early stages and survival of both fingerlings and juveniles. On the other hand, the FCR and growth rate in juveniles found in this study contrast with those from previous work, evidencing improved response under probiotics inclusion. Estimates for the SGR obtained here are higher than those previously reported, as a result of different genetic background and rearing conditions, which complement key factors such as probiotics species, target benefit (health, growth) and dosages.
SUMMARY
Samples of digestive systems of juvenile cobia were analyzed for metabolites profiles in order to detect potential modulation processes induced by probiotics at the gastro-intestinal level. Gas chromatography (GC) and capillary electrophoresis (CE) results evidenced similar profiles between groups for fatty acids and cations, respectively, whereas no values were detected for organic acids of low molecular weight. With regard to the free amino acid profiles, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) assessments revealed similar amino acid composition for both experimental groups in each pulse period. However, the concentration of each amino acid increased as a function of time in the supplementation group, whereas the control group showed an opposite tendency. The total free amino acid concentration (ppm) at the end of the of the experiment was 1.5 and 2.8 times higher than in the control group, as evidenced by spectrophotometry and HPLC analysis, respectively. This increased concentration could be related to higher free amino acid availability from food hydrolysis modulated by probiotics; however, the effects of such increment appear to be negligible regarding feed conversion and growth.

BACKGROUND
Lipids and their constituent fatty acids are, along with proteins, the major organic constituents of fish, and they play major roles as sources of metabolic energy for growth,
reproduction and movement (Tocher, 2003). Pelagic, carnivorous fish such as cobia require high levels of protein (45%) and fat (5–15%) for optimal commercial production (Fraser and Davies, 2009). Digestive processes drive the protein and lipid catabolism through the breakdown of proteins into free amino acids (which may be directly recycled, used to make new amino acids, or undergo catabolism via the Krebs cycle) and through the lysis of triglycerides into free fatty acids (which will be up-taken by the enterocytes in the intestinal mucosa; (Tocher, 2003)). Lipids produce energy in the form of adenosine triphosphate (ATP) synthesis, whereas fatty acids are important for energy storage, phospholipid membrane formation, and signaling pathways.

The intestinal microbiota is involved in the utilization and catabolism of several amino acids originating from both alimentary and endogenous proteins. These amino acids can serve as precursors for the synthesis of metabolic end products expressed by the microbiota including short chain fatty acids; since microbiota also provides the host with amino acids, gut bacteria can modify the amino acids bio-availability in the systemic circulation though both ways. (Neis et al., 2015). Experimental evidence suggests that bacteria in the small intestine play an active role in the metabolism of lysine, threonine, arginine, glutamate and glutamine. (Dai et al., 2011; Metges and Petzke, 2005). Cations and organic acids of low molecular weight (e.g. propionic, lactic, calcium formate, sodium formate) on the other hand, acidify the gastro-intestinal environment, improving digestion conditions and inhibiting gram-negative bacteria, while providing complementary energy sources (Lückstädt, 2008).

This work assessed the effects of pulse-probiotics inclusion (10^9 UCF kg^{-1} dry feeds) on the profiles of key metabolites measured in digestive systems of juvenile cobia,
using samples from a related study in which juvenile cobia under probiotic supplementation did not evidence improved aquaculture performance in terms of growth, feed conversion ratio, survival and ammonia excretion. The digestive system samples obtained during that experiments were either lyophilized or fixed with ethanol, and shipped to the GIEM laboratory in Universidad de Antioquia (Colombia) for posterior analysis; a variety of analytical methods were used for estimating the metabolites concentrations in order to detect potential modulation processes mediated by the probiotics supplementation.

MATERIAL AND METHODS

**Experimental design, sampling periods and measured variables.** Sampling times matched the end of each pulse probiotics period: 1) at 81 DPH, after 15 days of probiotic supplementation, 2) at 89 DPH, after 8 days of probiotics withdrawal and 3) at 96 DPH, after 7 additional days of probiotics supplementation. In each sampling period, four fish from each group replicate were randomly selected and lethally anesthetized (Eugenol at 40 ppm) for removing their whole fish digestive systems. The variables measured in the laboratory were concentration of cations, organic acids of low molecular weight, fatty acids and amino acids. Samples for cations, fatty acids and organic acids analysis were lyophilized and ground, whereas the samples for amino acid were fixed in absolute alcohol and blended with a medical grade tissue homogenizer.

**Laboratory analysis - Cations and organic acids of low molecular weight profiles.** Cations (total potassium, calcium, sodium, magnesium and zinc) and organic acids of low molecular weight (propionic, aldonic, formic, acetic and oxalic) were
determined by capillary electrophoresis (CE) using a Hewlett Packard Capillary Electrophoresis 3DCE system and calibration curve made from a mix of the evaluated cation standards. Lyophilized samples from each group replicates were pooled and 1.5-2.0 g were used for the analysis. 35 mL of aqua regia (HCl : HNO₃; 3:1) were added to each of the pooled samples and slowly evaporated until complete dryness, repeating this process with an additional 15 mL of aqua regia. Once the volumetric flask was cold, 40 mL of HPLC-grade water were added and this volume was filtered with qualitative paper filter, which was also rinsed with HPLC-grade water. The filtered solution was completed to 50 mL with HPLC-grade water, from which a dilution of 2 mL in 25 mL was evaluated by CE. Results are reported as: Cation or organic acid (%) = 0.125 x analyte concentration (from the curve) / g of sample. Each pooled sample was analyzed by duplicate, calculating its mean, which was used for assessing the concentration using the calibration curve previously obtained.

**Fatty acid methyl esters (FAMEs) profiles.** Ten fatty acids methyl esters (FAMEs) were assessed by gas chromatography using a gas chromatographer Agilent 6890N, equipped with a flame ionization detector (FID), a Split/ Split less injection system, an auto-injector Agilent 7683 and a ChemStation data processor. A mix of the ten-methyl esters was prepared using individual FAMEs stock solutions in a volumetric flask completing the final volume with n-heptane. The mix was prepared for having a final concentration of each analyte at: Methyl Laureate: 125 ppm Methyl Miristate: 100 ppm Methyl Palmitate: 500 ppm Methyl Estearate: 82 ppm Methyl Oleate: 136 ppm Methyl CLA (conjugated Linoleic Acid): 33.2 ppm Methyl Linoleate: 100 ppm Methyl Linolenate: 100 ppm Methyl Araquidonate: 100 ppm Methyl Docosahexanoate: 100 ppm.
Each sample was run by duplicate and the relative percentage was calculated based on the mean. The FAMEs content is expressed as the percentual composition of the analytes, referred to the total signals corresponding to analytes susceptible of esterification. All chromatogram signals (the peaks obtained after the n-heptane signal) are added to obtain the total area of esterification analytes. The area of each individual analyte is divided by the total area, in order to obtain the relative percentual composition of each analyte. 

\[ \text{Analyte} \% = \left( \frac{\text{Analyte area}}{\text{Total area}} \right) \times 100 \]

**Free amino acids profile Thin layer chromatography (TLC) essays.** Blended digestive systems samples were completed to 30 mL with methanol (reactive grade, 99%) and placed in a shaker for two hours. An aliquot of 10 mL was transferred into a glass test tube and centrifuged for 15 min at 6,000 RPM. Two L of the supernatant from each sample were ran on a one dimension thin layer chromatography plate (1D-TLC) for a visual profile assessment in each of the evaluated periods; a Merk stationary phase of silica gel 60 F254 and mobile phase of 2-butanol : acetic acid : water (3:1:1) was used. Subsequently, the replicates in each group for each of the three sampled periods were pooled and concentrated in a rotary evaporator (Buchi Rotavapor RE 111 / 461 water bath) using previously weighted volumetric flask. The mass obtained in each extract was calculated and the extract was re-suspended in 10 mL methanol; two L were ran in 1D-TLC along with a mix of eight amino acid standards (ALA, CYS, GLY, LEU, LYS, MET, TYR and VAL).

Subsequently, pooled samples were analyzed by two-dimension thin layer chromatography (2D-TLC) for further amino acid profiles separation, using the same stationary phase and two mobile phases: 2-propanol (28): methanol (8): ammonium (7):
water (7) and 2-butanol (35) : acetone (35) : acetic acid (7) : water (23). The retention factor (Rf) of ten amino acids standards (ALA, CYS, GLU, GLY, LEU, MET, PHE, SER, TRP and VAL,) was estimated first in 1D-TLC and then in a 2D-TLC using a mix of standards with each of both mobile phases.

**Spectrophotometry.** Total free amino acid concentration was estimated by the Ninhydrin method described using ethanol instead of 2 methoxyethanol for dissolving the Ninhydrin and the stannous chloride. Ninhydrin decarbolxylates the alpha-amino acids yielding an intensely bluish purple product that can be calorimetrically measured at 570 nm. Glycine standard solutions were prepared at 20, 40, 60, 80 and 100 ppm and mixed with Ninhydrin; each standard was run by duplicate in the spectrophotometer to obtain the calibration curve and the equation for estimating the amino acid concentration in each sample. Glycine standards concentration accounted for 95.8% of the variation in absorbance (R2=95%) with a statistically significant regression model (F(1,97) = 14.39, p< 0.0005) yielding a prediction equation of ppm = (ABS + 0.13) / 0.06.

Samples were run in the spectrophotometer by duplicate using a dilution factor of 0.01, and their concentrations (ppm) estimated with the equation obtained form the calibration curve. The mean value obtained from the replicates was then multiplied by the dilution factor and by the volume in which the extract was re-suspended (0.01 L), dividing this results by the extract mass to obtain the total free amino acid concentration ppm (mg L\(^{-1}\)). A linear regression analysis was run for time (DPH) and total amino acid concentration in each group to test for significant linear association between variables, using a significance of 0.1 due to the reduced number of observations used to fit the model.
**High performance Liquid chromatography (HPLC).** Quantitative analysis of 14 amino acids was performed with derivatization using in an HPLC Thermo Ultimate 3000 using a ZORBAX Eclipse AAA (4.6mm x 75 mm (3.5 m)) column, with mobile phases A: A: 40 mM NaHPOR pH 7.8 [5.5 g NaH PO₄ monohydrate + 1 liter water, adjust to pH 7.8 with NaOH solution (10 N)] and B: ACN: MeOH: water (45:45:10, v/v/v). The pooled samples, one from control and one from the probiotics treatment in each of the sampling periods were ran in HPLC; the molecular mass was obtained by multiplying the concentration of moles/mL by the dilution factor; this result was then multiplied by the volume of re-suspended extract (0.01 L) and divided by the mass obtained from the Rotavapor extract in order to get each amino acid concentration in ppm (mg L⁻¹).

RESULTS

**Organic acids of low molecular, cations and fatty acids profiles.** There were no differences in cations and fatty acids profiles between experimental groups (Figures 3.1 and 3.2, respectively) in the first and last evaluated sampling periods (81 and 96 DPH). Regarding the intermediate period (89 DPH), three out of four flasks containing the 89 DPH samples were damaged during the lyophilization process, resulting with a single data corresponding to probiotics treatment. Although it was not possible to compare the results with the control group for this intermediate sampling period, its concentration values for both, cations and fatty acids, are very similar to the those concentrations obtained in the first and last sampling periods, which suggests that probiotic supplementation (10⁹ CFU Kg⁻¹ of feed) did not affect the digestive system environment
regarding cations and fatty acid. Organic acids of low molecular weight on the other hand, were not detectable by capillary electrophoresis.

Figure 3.1. Cations profiles in experimental groups at 81 and 96 DPH

Figure 3.2. FAMEs profiles in experimental groups at 81 and 96 DPH
**Free amino acid profiles - Thin layer chromatography (TLC).** 1D-TLC plate revealed an identical profile consisting of nine amino acids for all replicates in both experimental groups for each of the three sampling periods (Figure 3.3). Results from pooled samples also showed identical profiles in each of the evaluated periods (Figure 3.4). Five amino acids were identified as LYS, GLY ALA, VAL, and or LEU. Further amino acid profile separation through a 2D-TLC revealed 13 amino acids present in each of experimental groups, showing four additional amino acid that were not observed in the 1D-TLC. Of these 13 amino acids found in the experimental groups, seven to eight amino acids were identified through the migration patterns of the standards present in the mix (ALA, SER/GLU, VAL, MET, LEU, TRP and PHE) as there was an overlap of SER and GLU due to similar retention factors. The profiles obtained from this 2D-TLC were almost identical as well, suggesting no differences in the amino acid composition between experimental groups (Figure 3.5).

![Figure 3.3](image)

Figure 3.3. Amino acid variety in each group replicate obtained from a 1D-TLC for each of the evaluated periods
Figure 3.4. Left, 1D-TLC plate displaying the migration of the TYR standard, the mix of seven amino acid standards (LYS, GLY, ALA, VAL, MET, TRP, LEU) and the pooled samples for each experimental group, in which S1, S2, S3 refer to the sampling periods (at 81, 89 and 96 DPH, respectively); C refer to control group and P refer to Probiotics group. Right, corresponding Rf values for each amino acid in the standards mix.

<table>
<thead>
<tr>
<th>Position</th>
<th>Rf</th>
<th>Amino acid in standards mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.118</td>
<td>LYS</td>
</tr>
<tr>
<td>2</td>
<td>0.312</td>
<td>GLY</td>
</tr>
<tr>
<td>3</td>
<td>0.371</td>
<td>ALA</td>
</tr>
<tr>
<td>4</td>
<td>0.505</td>
<td>VAL</td>
</tr>
<tr>
<td>5</td>
<td>0.543</td>
<td>MET</td>
</tr>
<tr>
<td>6</td>
<td>0.624</td>
<td>TRP/LEU</td>
</tr>
<tr>
<td>7</td>
<td>0.597</td>
<td>TYR</td>
</tr>
</tbody>
</table>

**Spectrophotometry.** Total free amino acid concentration was higher for control group in the first and second evaluated periods, whereas in the probiotics group it increased as a function of time, being higher than the control group in the third evaluated period (Figure 6). The linear regression showed that, even though DPH accounted for the 95% of the variation in total amino acid concentration in the probiotics group, the significance of the slope coefficient was right at the boundary of statistical significance (R2=0.95; F(1,1)=38.15; p=0.10), whereas in the control the linear association between variables was not statistically significant (R2=0.52; F(1,1) = 1.07; p = 0.49).
Figure 3.5. Left, 1D-TLC and 2D-TLC amino acid standards. Right, Amino acid profiles obtained from 2D-TLC; control group is on the left and probiotics on the right; upper, sampling period 1; middle, sample period 2; bottom, sampling period 3

Figure 3.6. Total free amino acid concentration after each of the probiotics pulsed-supplementation period estimated by spectrophotometry
High performance liquid chromatography (HPLC). Results from HPLC confirmed the findings obtained with both, TLC and spectrophotometry, regarding similar amino acid composition profiles, but different total amino acid concentrations between control and probiotics group. Interestingly, each one of the 14 amino acids assessed by HPLC increased its concentration as a function of time in the probiotics group compared to the control group (Figures 3.7 and 3.8). The trend displayed by the HPLC results was similar to that obtained by spectrophotometry; however, values are lower than in spectrophotometry most likely due to the higher sensitivity of the latter, in which all amino acids react with the Ninhydrin.

![Total free amino acid concentration (ppm) - HPLC](image)

**Figure 3.7.** Concentration (ppm) of total free amino acid concentration (right) obtained at the end of each probiotics pulsed-supplementation measured by HPLC.
Figure 3.8. Individual concentration (ppm) of 14 amino acids measured by HPLC
DISCUSSION

During the digestion process, proteins are hydrolyzed to shorter polypeptide chains or free amino acids, digestible carbohydrates to simple sugars, and lipids to fatty acids and glycerol. Some amino acids and their metabolites regulate key metabolic pathways that affect maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction and immune response; in aquatic animals, recent evidence suggests that taurine, glutamine, glycine, proline and hydroxyproline promote growth, development, and health, driving the feeds industry to supplement the diets with particular amino acids which might increase the chemo-attractive property and nutritional value of aqua feeds with low fishmeal inclusion, optimize efficiency of metabolic transformation in juvenile and sub adult fishes, suppress aggressive behaviors and cannibalism, increasing larval performance and survival; regulate spawning timing, improve taste and texture and enhance immune system and tolerance to environmental stressors (Li et al., 2009).

On the other hand, cations and organic acids of low molecular weight such as formic, acetic, propionic, lactic, citric, calcium formate, sodium formate, calcium propionate and calcium lactate, have been used as acidifiers in fish nutrition to improve overall performance in both, cold-water and tropical species. These compounds lower the pH and inhibit bacterial growth in both, dry feeds and fish gastro-intestinal tract improving the conditions for digestive processes; Also, the organic acids represent an energy source, as they are generally absorbed through the intestinal epithelia by passive diffusion and they can be used in various metabolic pathways for energy generation (e.g. ATP generation in the citric acid cycle; Lückstädt (2008)). The assessment of short fatty
acids by HPLC has been used as a proxy for lactosucrose fermentation in the digestive system of sea bream (*Pagrus major*), obtaining that fish fed a diet supplemented with lactosucrose showed a significantly higher concentration of short fatty acids in the intestine (Kihara, 2008). With respect to cobia in particular, the dietary levels of protein and fatty acids, as well as the effects of supplementation (e.g. dietary compounds, probiotics) have been addressed by number of studies (Trushenski et al., 2012; Chou et al., 2001; Faulk and Holt, 2005; Geng et al., 2011, 2012; Garrido-Pereira et al., 2014; He, 2015). However, there are not previous reports in teleost fish species using a similar methodological approach as the one used in the present study for assessing the dynamics of key metabolites in digestive system.

Results from capillary electrophoresis (CE) and gas chromatography (GC) showed similar profiles between experimental groups for cations and fatty acids, respectively, whereas CE in any of the samples did not detect organic acids of low molecular weight. With regard to the free amino acid profiles, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) assessments revealed similar amino acid composition for both experimental groups in each pulse period. However, from a quantitative perspective, results revealed that the concentration of each amino acid increased as a function of time in the supplementation group, whereas the control group showed an opposite tendency. The total free amino acid concentration (ppm) at the end of the experiment was 1.5 and 2.8 times higher than in the control group, as evidenced by spectrophotometry and HPLC analysis, respectively. This increased concentration could be related to a higher free amino acid availability from food hydrolysis modulated by probiotics, as evidenced in other groups of vertebrates (Dai
et al., 2011; Metges and Petzke, 2005); however, the effects of such increment appear to be negligible regarding feed conversion and growth. Based on these results, it is likely that a higher probiotics inclusion proportionally increases the total free amino acid concentration in the digestive tract with potential effect on growth or feed conversion once this concentration surpasses certain threshold. The methodological approach followed in this study for the assessment of key metabolites in the digestive system could be used in further research evaluating other probiotics supplementation (e.g. continued supplementation at higher dosages), which could be also coupled with HUFAs and or organic acids inclusion.

CONCLUSION
Profiles for both, cations and fatty acid, obtained from capillary electrophoresis (CE) and gas chromatography (GC), respectively, were very similar for control and probiotics in each of the sampling periods, evidencing minimal changes over the probiotics-pulsed supplementation. CE did not detect organic acids of low molecular weight in any of the sampling periods. With regard to the free amino acid profiles, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) assessments revealed similar amino acid composition for both experimental groups in each pulse period. However, from a quantitative perspective, results revealed that the concentration of each amino acid increased as a function of time in the supplementation group, whereas the control group showed an opposite tendency. The total free amino acid concentration (ppm) at the end of the experiment was 1.5 and 2.8 times higher than in the control group, as evidenced by spectrophotometry and HPLC analysis, respectively. This
increased concentration could be related to a higher free amino acid availability from food hydrolysis modulated by probiotics; however, the effects of such increment appear to be negligible regarding feed conversion and growth as evidenced by the aquaculture performance results obtained before the analysis of gut samples. The methods considered in this study for assessing key metabolites at the digestive tract level could be used in further research addressing other types of supplementation (e.g. fatty acids, amino acids, organic acids) besides of probiotics.
CHAPTER 4:
PROBIOTICS AND 1-MONOGLYCERIDES AS FEED SUPPLEMENTS FOR YELLOWFIN TUNA (Thunnus albacares) AT EARLY DEVELOPMENTAL STAGES

SUMMARY
The effects of probiotics and 1-monoglycerides supplementation on the growth and survival of yellowfin tuna larvae were evaluated at 9-10 DPH, a common age of acute mortality largely due to nutritional deficiencies. Two probiotic trials (carried out at different stocking density and different probiotic inclusion) and one trial with 1-monoglycerides were performed. The higher probiotic supplementation (40x10^6 CFU L^-1; Trial 2) yielded a significantly higher total length in the supplementation group with respect to the control in 10 DPH YFT larvae, with no significant differences in survival between groups for both trials. With regard to 1-monoglycerides, no significant differences in both variables were found either, although the supplementation group yielded a two-fold increase in survival compared to the control group, and overall, had the higher and less variable values. Probiotics might have assisted digestive processes of the rudimentary digestive system of pre-flexion larvae, whereas 1-monoglycerides might have contributed as additional energy sources, both enabling higher larval nutrient uptake and growth.

BACKGROUND
Tunas and related scombrid species play fundamental roles in the pelagic food webs of the world's tropical and temperate waters and represent one of the most valuable
fisheries, representing over 10% of the global capture with estimated end value of US$ 42 billion (Galland et al., 2016). Average adult biomass of large tunas (bluefin, bigeye and yellowfin) have declined 62.8% in the last 50 years, with most fisheries currently considered as fully exploited or overexploited (Juan-Jorda et al., 2011). Of the eight species of true tunas, the International Union for Conservation of Nature (IUNC) considers one as critically endangered (Southern bluefin Thunnus maccoyii), one as endangered (Atlantic bluefin T. thynnus), two as vulnerable (bigeye T. obesus and Pacific bluefin T. orientalis), two as near threatened (yellowfin T. albacades and albacore T. alalunga), one as least concern (blackfin T. atlanticus) and one (longtail T. tonggol) without sufficient data (Collette et al., 2011f,d,a,h,b,c,e,g).

As price and demand for tunas continue to increase, farming approaches for the most valuable tuna species are transitioning from capture-based operations to closed-cycle aquaculture production. Ranching and fattening practices have developed exponentially in the last decade in the Mediterranean region, Mexico, Australia and Japan as a result of the rising demand for sashimi and sushi markets. Current operations grow Atlantic bluefin tuna, Pacific bluefin tuna and Southern bluefin tuna. Yellowfin tuna was also farmed in Mexico, but issues related with wild stock availability of proper size limited the industry (Buentello et al., 2016). To increase the size and fat of a large proportion (17-37%) of wild caught bluefin tuna, the ranching and fattening sector is also using small pelagics (sardines, anchovies) as feed sources in floating cages, yet such practices are not deemed sustainable in the long term (Ottolenghi, 2008; Katagiri et al., 2017; Benetti et al., 2016; Metian et al., 2014; Mylonas et al., 2010). The aquaculture potential for tunas has been addressed for more than four decades with limited success,
until recently achieved milestones such as the closed-cycle production of bluefin tuna (BFT) by Japan and Spain and the first generations (F1s) of hatchery-produced yellowfin tuna (YFT) juveniles in Panama in cooperation with Japan (Zohar and Corriero, 2016; Sawada et al., 2005; Wexler et al., 2003; Benetti et al., 2016, Margulies et al., 2016).

Japan has been at the forefront of tuna production since the early 70's through the participation of universities and federal and prefectural governments (Masuma et al., 2011). The first experimental YFT larval rearing was conducted by Kinki University (now known as Kindai University) in 1969 from artificial fertilization using wild caught broodstock. After several years of unavailability of mature eggs, experiments resumed in 1976 (Harada, 1978) resulting in the first YFT fingerlings (38 DPH) artificially reared. Based on these preliminary results, the Japan Sea Farming Association (JASFA) started YFT seed production as a model for further research on BFT, and obtained the first successful larval rearing (beyond metamorphosis) in 1979. A BFT research project began in 1987 with the rearing of wild-caught juveniles, which spawned and produced the first generation of hatchery-produced BFT in 1995/1996. A second generation was produced in 2002 with 17,307 juveniles cultured in an open-sea cage, thus completing the life cycle under controlled conditions (Sawada et al., 2005). Between 2002 and 2007, about 10,000 fingerlings were produced annually. By 2009, 45,000 fingerlings were being produced per year, and over 265,000 by 2012.

Despite the significant increase in fingerling production, mass mortality (at early stages of development, just after first feeding, about at 10 DPH and around transformation to the juvenile phase) represents a major constraint for the tuna aquaculture industry (Kaji, 2000; Sawada et al., 2005; Katagiri et al., 2017). Similarly,
the first spontaneous spawning of YFT occurred in 1992 and multiple rearing trials for YFT larvae and juveniles have been performed since then. Also, since 1996 the Inter-American Tropical Tuna Commission maintains a continuously-spawning population of YFT in a land-based facility in Panama, providing opportunities for research on courtship and spawning behavior, early stages of development, and a recent experience of hatchery-reared juveniles transferred into an experimental floating cage (Margulies et al., 2007; Benetti et al., 2016, Margulies, 2016). As in BFT, survival rates of YFT remain low due to high mortality at two particular stages: a few days after the onset of the exogenous feeding and during the transformation phase to juveniles. The onset of the exogenous feeding is a critical stage of high mortality since the larvae depend on external feed sources of appropriate abundance, size and nutrient composition to sustain their high metabolic requirements (Kaji, 2000; Margulies, 2016). A number of studies have addressed the low survival rate at early development in BFT (Ishibashi et al., 2014; Honryo et al., 2013; Kurata et al., 2012; Koven et al., 2018; Biswas et al., 2006; Okada et al., 2014; De la Serna et al., 2010; Seoka et al., 2008, 2007; Higuchi et al., 2014; Katagiri et al., 2017) and in YFT (Partridge et al., 2011; Katagiri et al., 2017; Buentello et al., 2011; Kaji et al., 1999b,a; Margulies, 1993; Margulies et al., 2007, Margulies et al., 2016).

Research on feed supplementation has shown that probiotics and short-chain fatty acids have positive effects in the aquaculture performance of several species. Short-chain and medium-chain 1-monoglycerides are composed of a molecule of glycerol linked to a fatty acid. In recent years, supplementation with these compounds in both terrestrial (poultry, swine, ruminants, rabbits) and aquatic farmed species, have shown selective
antibacterial action in digestive systems, improved nutritional effects and emulsifying effect in digestion of other dietary lipids and protein, resulting in concomitant effects on growth, survival and feed conversion efficiency. The benefits of 1-monoglycerides inclusion in aquaculture have been demonstrated in a number of species such as white leg shrimp (*Litopenaeus vannamei*), white sturgeon (*Acipenser transmontanus*) and carp (*Cyprinus carpio*), and Japanese yellowtail (*Seriola quinquergiata*) (Parini, 2016). Both probiotics and fatty acids can be administered to the rearing water or mixed with the feeds, using either a single composition or a mixture of synergistic types (Van Hai, 2015; Douillet, 2000; Zink et al., 2011; Parini, 2016). In the case of marine pelagic species that prey on zooplankton during early stages of development, such as YFT, these supplements are added to the enrichment food for live feeds (e.g. rotifers, *Artemia* sp.).

This study evaluated the effects of enriched rotifers supplemented with both, probiotic and 1-monoglycerides, on the growth and survival of 9-10 DPH yellowfin tuna larvae, a common age for acute mortalities during the pre-flexion - flexion transition. One experiment using 1-monoglycerides supplementation and two experiments with probiotics inclusion, carried out at different stocking densities and different probiotics dosages, were performed at the facilities of the Inter-American Tropical Tuna Commission (IATTC)-Achotines Laboratory (Pacific coast of Panama).

**MATERIALS AND METHODS**

*Dietary supplements, rotifer enrichment and feeding schedule.* Enriched rotifers (Ori-one®; Skretting, Norway) were supplemented either with probiotics or with 1-Monoglycerides before feeding the YFT larvae. The probiotic used was a liquid bacterial
blend (*Bacillus subtilis*, *B. lincheniformis*, *B. magaterium*, and *Brevibacillus laterosporus* at a concentration of 1x10^8 CFU mL^-1; Eco Microbials, LLC) and the 1-monomglycerides product contained a mixture of short and medium-chain fatty acids (Propionic, butyric, caproic, heptanoic, caprylic, nonanoic, capric and lauric acid) esterified with glycerol in powder form; SILOhealth108P®; SILO, Italy). Mass cultures of rotifers were reared in two plastic tanks of 1,000 L and fed with a mix of yeast (Levapan®0.3-0.5 g for each 10^6 rotifers day^-1 split into four rations) and live microalgae *Nannochloropsis* sp. (30-50% tank volume day^-1). The rotifers required for feeding the YFT larvae were harvested daily from the mass cultures and rinsed with filtered / UV seawater for 30-40 minutes. After this cleanup, rotifers were transferred into two enrichment tanks of 100 L, one assigned to the control group and the other to the supplementation group. Rotifers in both units were enriched overnight for 12 hours (0.3-0.5 g Ori-one® for every 10^6 rotifers, mixed with 3 L of water and blended for five minutes). In the case of the enrichment tanks receiving supplementation, 1-monomglycerides product was blended along with Ori-one, whereas probiotics were added to the enrichment solution after blending. Enrichment solutions (control and supplementation) were fed the rotifers at three rations, 1 L every four hours.

In the probiotics trials, rotifers were supplemented with 19x10^6 CFU L^-1 and 4019x10^6 CFU L^-1 for Trial 1 and Trial 2, respectively, whereas the rotifers used in the 1-Monoglycerides trial were supplemented with SILOhealth108P® at 4% of the total feed (Ori-one®). After 12 hours of enrichment and supplementation with either Probiotics or 1-monomglycerides, rotifers were harvested, cleaned up and transferred into coolers set up with gentle aeration and oxygen supply. The rotifer population was estimated by counting and averaging three samples of 0.1 mL, extrapolating to the cooler
volume. YFT larvae were fed three times a day (6:30, 11:30 and 3:30 hours). Before each feeding, the residual rotifer density in the larval rearing tanks was estimated by counting and averaging the rotifers present in four 200 mL samples, extrapolating to the larval rearing tank volume. The amount of rotifers required for maintaining a particular rotifer density (Table 1) was estimated and scooped out from the rotifer coolers. After delivering the first feeding of the day, ice bottles were used inside the coolers to keep the temperature low (10-12° C) and reduce the catabolism of enriched rotifiers.

**Collection of eggs.** Fertilized eggs were collected from volitional spawning events by YFT broodstock of the IATTC-Achotines Lab (Wexler et al., 2003; Margulies et al., 2016) between 00:00 and 02:30 hours. After an estimation of the fertilization ratio and total egg number, eggs were incubated in 160 L conical tanks. Larvae hatched after approximately 24 hours and were volumetrically estimated and equally distributed into the experimental tanks at 2 DPH.

**Table 4.1.** Rotifer density (rotifers mL$^{-1}$) daily maintained in larval rearing tanks during supplementation experiments

<table>
<thead>
<tr>
<th>DPH</th>
<th>Probiotics</th>
<th>1-Monoglycerides (SILOHealth®)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>9</td>
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<tr>
<td>7</td>
<td>6</td>
<td>9</td>
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<tr>
<td>8</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>10</td>
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<tr>
<td>10</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

**Experimental setup and variables.** The experiments tested a control group and a supplementation group using either three or four replicates randomly assigned. These experimental units consisted of circular, fiberglass tanks ranging from 700 to 1,000 L, set
up with a flow-through system (200-300% daily water exchange), gentle aeration and a photoperiod of 12 hours light:12 hours dark cycle. Four fluorescent light bulbs at 50 cm of the water surface were used for light consistency from 6:00 to 18:00 hours, covering them with a shade-cloth from 6:00-7:00 and from 17:00-18:00 to emulate sunrise and sunset conditions. Live algae (*Nannochloropsis oculata*) were added periodically to the larval rearing tanks to maintain a cell density between 0.5 and 1.0x10^6 cells mL\(^{-1}\) (Partridge et al., 2011; Margulies et al., 2016). Dissolved oxygen ranged between 5.4 and 6.05 mg L\(^{-1}\); temperature between 27.1 and 28.6° C; salinity between 30.6 and 31.5 ppt and pH between 8.03 and 8.27. The variables evaluated in these trials were survival (total number of YFT larvae obtained at the end of each experiment) and growth at 9-10 DPH. Total length (TL) was assessed in the three experiments, measuring 10-20 YFT larvae in each replicate with a micrometer set up in a microscope. Dry weight (DW) also was measured with a microscale in the 1-monoglycerides experiment, following the method described by Margulies et al. (2007) and Katagiri et al. (2017). Survival was assessed by concentrating the volume of the tanks and individually counting the YFT using plastic jars and hand tally-counters. In each of the experiments, mean values for either TL or DW were calculated for each of the replicates before running the statistical analyses in order to avoid pseudo-replication.

**Probiotic trials.** Two experiments were carried out, each using different stocking densities and probiotics supplementation levels. Trial 1. Three replicates consisting of 700 L tanks were evaluated in each treatment using a stocking density of 18 YFT larvae L\(^{-1}\) and a probiotics supplementation of 19x10^6 CFU L\(^{-1}\). Total length (mm) was sampled at 7 and 10 DPH, measuring 20 YFT larvae in each replicate. Trial 2. Four replicates
consisting of 700 L tanks were evaluated in each treatment using a stocking density of 40 larvae L\(^{-1}\) and a probiotics supplementation of 40x10\(^6\) CFU L\(^{-1}\). Total length (mm) and dry weight (g) were sampled at 10 DPH, measuring 20 YFT larvae in each replicate.

**1-Monoglycerides trial (SILOhealth®).** One trial with 1-Monoglycerides was carried out using three replicates per treatment consisting of 1,000 L tanks, a stocking density of 22 YFT larvae L\(^{-1}\) and a supplementation level of SILOhealth108® equivalent to 4% of the enrichment feed. Total (mm) length and dry weight (g) were sampled at 9 DPH, measuring 10 YFT larvae in each replicate.

**Statistical analysis.** Data sets from each variable were checked for outliers, normality and homoscedasticity and tested for differences between groups, either with parametric or non-parametric analyses using the software SPSS24® (IBM®) and a significance level of \(\alpha=0.05\). Final survivals in each replicate tank were not adjusted for sample removals. In probiotics Trial 1, both variables, TL and survival, were evaluated with an independent samples t-test. In probiotics Trial 2, a Mann-Whitney U-test was used to evaluate TL whereas an independent t-test was used for assessing survival. With regards to the 1-Monoglycerides trial, both variables, TL and survival, were assessed with independent samples t-tests. Data are mean ± standard deviation, unless otherwise stated.

RESULTS

**Probiotics Trial 1. (18 YFT larvae L\(^{-1}\); 19x10\(^6\) CFU L\(^{-1}\)) - Growth at 7 and 10 DPH.** In both of the sampled ages, 7 and 10 DPH, the independent samples t-test determined no significant differences between control and probiotics groups. At 7 DPH,
the mean TL for control and probiotics group was 3.92 ± 0.080 and 3.89 ± 0.036, respectively, with a mean difference of 0.03 (95% CI, -0.110 to 0.170) not statistically significant, t(4) = 0.592, p = 0.586. At 10 DPH, the corresponding values for control and probiotics were 4.54 ± 0.113 and 4.34 ± 0.107, respectively, with a mean difference of 0.2 (95% CI, -0.051 to 0.451) not statistically significant, t(4) = 2.21, p = 0.092 (Table 4.2; Figure 4.1).

Table 4.2. Group statistics and independent samples t-tests output for total length (mm) in probiotics Trial 1

<table>
<thead>
<tr>
<th></th>
<th>7 DPH</th>
<th>10 DPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Probiotics</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.92 ± 0.080</td>
<td>3.89 ± 0.036</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.046</td>
<td>0.020</td>
</tr>
<tr>
<td>Levene’s test sig.</td>
<td>0.396</td>
<td>0.953</td>
</tr>
<tr>
<td>Mean diff.</td>
<td>0.030</td>
<td>0.020</td>
</tr>
<tr>
<td>t(4)</td>
<td>0.592</td>
<td>0.586</td>
</tr>
<tr>
<td>Sig.</td>
<td>0.586</td>
<td>0.092</td>
</tr>
<tr>
<td>95% C.I. of the diff.</td>
<td>-0.110 to 0.170</td>
<td>-0.051 to 0.451</td>
</tr>
</tbody>
</table>

Figure 4.1. YFT total length (mm) at 7 and 10 DPH in the probiotics Trial 1
**Survival.** The mean number of fish at 10 DPF trended higher in the probiotics group (1,079.3 ± 1,153.5) than in the control group (805.3 ± 799.2) but due to high variances among replicate tanks, the mean difference of -274.0 (95% CI, -2,523.5 to 1,975.5) was not statistically significant, t(4) = -0.338, p = 0.752 (Table 4.3, Figure 4.3). In the control group, survival ranged from 0.3 to 13% whereas in the probiotics group, it ranged from 1.1 to 18.8%. A total of 5,654 10 DPH YFT larvae were produced between both treatments, with the control group representing 43% of the total (Table 4.5) and the probiotics group representing 57%.

**Table 4.3.** Group statistics and independent samples t-tests output for survival (total 10 DPH YFT larvae) in probiotics trials 1 and 2

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Probiotics</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>805.33 ± 799.21</td>
</tr>
<tr>
<td>S.E.</td>
<td>461.42</td>
</tr>
<tr>
<td>Levene's test sig.</td>
<td>0.456</td>
</tr>
<tr>
<td>Mean diff.</td>
<td>-274.0</td>
</tr>
<tr>
<td>t(4-6)</td>
<td>-0.338</td>
</tr>
<tr>
<td>Sig.</td>
<td>0.752</td>
</tr>
<tr>
<td>95% C.I. of the diff.</td>
<td>-2523 to 1975.52</td>
</tr>
</tbody>
</table>

**Trial 2.** (40 YFT larvae L⁻¹; 40×10⁶ UFC L⁻¹) - Growth at 10 DPH. Mean larval TL in the probiotics group (4.4 0.08 mm) was higher than in the control group (4.15 0.321 mm). The Mann-Whitney U test showed that distributions of the TL scores for control and probiotics groups were not similar, as assessed by visual inspection. TL scores for the probiotics group (mean rank = 6.5) were statistically significantly higher than those for the control group (mean rank = 2.50), U=0.000, Z=-2.35, p=0.029 (Table 4.4; Figure 4.2)
Table 4.4. Length ranks and statistic output from the Mann-Whitney U test performed for the YFT in Trial 2 (40 larvae L\(^{-1}\) and 4 \times 10^6 CFU L\(^{-1}\))

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean rank</th>
<th>Sum of ranks</th>
<th>Mann-Whitney U</th>
<th>Test Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>2.50</td>
<td>10.0</td>
<td>Z</td>
<td>-2.35</td>
</tr>
<tr>
<td>Probiotics</td>
<td>4</td>
<td>6.50</td>
<td>26.0</td>
<td>Asymp. Sig.</td>
<td>0.019</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td></td>
<td></td>
<td>Exact Sig.</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Figure 4.2. YFT total length (mm) at 10 DPH in the probiotics Trial 2

Survival. The mean number of fish at 10 DPH trended higher in the probiotics group (133.2 ± 64.6) than in the control group (96.0 ± 40.6), but the mean difference of -37.25 (95% CI, -130.65 to 56.15) was not statistically significant, \(t(6) = -0.976, p = 0.367\) (Table 4.3; Figure 4.3). In the control group, survival ranged from 0.2 to 0.5% whereas in the probiotics group, it ranged from 0.2 to 0.8%. A total of 917 10 DPH YFT larvae were produced between both treatments, with the control group representing 42% (Table 5) of the total and the probiotics group representing 58%.
Figure 4.3. Final number of 10 DPH YFT obtained for both probiotics trials

Table 4.5. Final survival (Total count (TC) and percentage survival) of 9-10 DPH YFT in each of the experimental units at the end of the supplementation trials

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tank Replicate</th>
<th>Probiotics Trial 1</th>
<th>Probiotics Trial 2</th>
<th>1-Monoglycerides (SILOHealth®)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TC %</td>
<td>TC %</td>
<td>TC %</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>745 5.9</td>
<td>135 0.5</td>
<td>352 1.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>38 0.3</td>
<td>58 0.2</td>
<td>1,917 8.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,633 13.0</td>
<td>127 0.4</td>
<td>203 0.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>- -</td>
<td>64 0.2</td>
<td>- -</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td>2,416</td>
<td>348 13.0</td>
<td>1,113 4.9</td>
</tr>
<tr>
<td>Supplementation</td>
<td>1</td>
<td>135 1.1</td>
<td>214 0.8</td>
<td>3,238 3.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2,395 18.8</td>
<td>127 0.4</td>
<td>1,797 8.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>738 6.9</td>
<td>135 0.5</td>
<td>2,166 9.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>- -</td>
<td>56 0.2</td>
<td>- -</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td>3,238</td>
<td>533 13.0</td>
<td>5,076 3.9</td>
</tr>
<tr>
<td>TOTAL survival</td>
<td></td>
<td>5,054</td>
<td>917 13.0</td>
<td>7,528 3.9</td>
</tr>
</tbody>
</table>

SILO-health®(1-monoglycerides) supplementation - Growth (TL and DW) at 9 DPH. No significant differences between groups were found in both growth variables, TL and DW. Mean TL was slightly higher in the SILOhealth® group (4.26 ± 0.27 mm) than in the control group (4.22 ± 0.21 mm) with a mean difference of -0.0367 not statistically significant (95% CI, -0.588 to 0.515; t(4)=-0.184; p=0.86), whereas mean DW was higher in the control group (0.1086 ± 0.018 g) than in the SILOhealth® group.
(0.0813 ± 0.014 g) with a mean difference of 0.027 not statistically significant (95% CI, -0.010 to 0.065; t(4)=2.06; p=0.109; Table 4.6, Figure 4.4).

**Table 4.6.** Group statistics and independent samples t-test output for total length (mm) and dry weight (g) at 9 DPH in the SILO-health (1-monoglycerides) trial

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SILO-health</th>
<th>Control</th>
<th>SILO-health</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>4.22 ± 0.21</td>
<td>4.26 ± 0.27</td>
<td>0.1086 ± 0.018</td>
<td>0.0813 ± 0.014</td>
</tr>
<tr>
<td><strong>S.E.</strong></td>
<td>0.12</td>
<td>0.16</td>
<td>0.0105</td>
<td>0.0080</td>
</tr>
<tr>
<td>Levene’s test sig.</td>
<td>0.739</td>
<td></td>
<td>0.652</td>
<td></td>
</tr>
<tr>
<td>Mean diff.</td>
<td>-0.037</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(4)</td>
<td>-0.184</td>
<td>2.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig.</td>
<td>0.86</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% C.I. of the diff.</td>
<td>-0.588 to 0.515</td>
<td>-0.010 to 0.065</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.4.** Yellowfin tuna total length and dry weight at 9 DPH in 1-Monoglycerides trial (SILOhealth®)

**Survival.** The mean number of fish produced was higher in the SILOhealth group and nearly double the mean number of fish produced in the control group (1,692 ± 308.5 vs. 824 ± 949.5). However, due to the small sample size of each treatment (n=3) and the high dispersion obtained in the control group, the independent samples t-test did not detect significant differences between supplementation and control (mean difference of -
868; 95% CI, -2,614.45 to 878.45 t(4) = -1.38, p= 0.24; Table 4.7; Figure 4.5). In the control group, survival ranged from 0.9 to 8.5% whereas in the SILOhealth group it ranged from 4.9 to 9.6%. A total of 7,548 9 DPH YFT larvae were produced between both treatments, with the control group representing 33% and the SILOhealth group representing 67% of the total (Table 5). Interestingly, the plots of survival and dry weight exhibited an inverse relationship, suggesting that the trend of higher dry weight obtained in the control group could be related to density dependent growth (Figure 5).

Table 4.7. Group statistics and independent samples t-test output for total number of 9 DPH YFT larvae in the SILO-health trial

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SILO-health</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>824 ± 949.5</td>
<td>1692 ± 308.5</td>
</tr>
<tr>
<td>S.E.</td>
<td>548.2</td>
<td>308.5</td>
</tr>
<tr>
<td>Levene’s test sig.</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Mean diff.</td>
<td>-868</td>
<td></td>
</tr>
<tr>
<td>t(4)</td>
<td>-1.38</td>
<td></td>
</tr>
<tr>
<td>Sig.</td>
<td>0.240</td>
<td></td>
</tr>
<tr>
<td>95% C.I. of the diff.</td>
<td>-2614.45 to 878.45</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.5. Effect of survival in mean dry weight for the 1-monoglycerides Trial

DISCUSSION

Among pelagic species, YFT stands out for its ecological and economic importance for fisheries and aquaculture potential. Despite major achievements towards closing the life cycle for this species, such as broodstock populations regularly spawning in controlled conditions and cage culture of F1 hatchery-reared YFT juveniles (Margulies et al., 2016), high mortality at early developmental stages continues to be a key bottleneck for scaled-production of fingerlings and juveniles. The first 10 days of BFT and YFT larval rearing constitute a critical mortality period, associated with sub-optimal nutrition at the beginning of the exogenous feeding, suboptimal physical conditions (light, microturbulence, water temperature), surface tension-related death (larvae become trapped within the water surface) and sinking syndrome caused by the hindering of swim
bladder inflation (Koven et al., 2018; Benetti et al., 2016; Honryo et al., 2016; Katagiri et al., 2017, Margulies et al., 2016). These issues have been addressed mostly in BFT species and to a lesser extent in YFT through a number of studies assessing morphological changes in digestive systems (Kaji et al., 1996, 1999a; Kaji, 2000), enzymatic activity and amino acid composition (Buentello et al., 2011), enrichment media for live feeds (Katagiri et al., 2017; Seoka et al., 2007, 2008; Koven et al., 2018; Biswas et al., 2006) and adjustment of physical variables such as photoperiod, aeration rates, light, microturbulence and water surface conditions (Partridge et al., 2011; Honryo et al., 2013, 2016; Margulies et al., 2016).

At the beginning of the exogenous feeding phase, marine fish larvae have absorbed most of the yolk sac and must prey on zooplankton of adequate size, abundance and nutritional profile to sustain their high metabolic and locomotion rates (Sawada et al., 2005; Kaji, 2000; Buentello et al., 2011). The functional digestive system of tuna larvae differentiates earlier than other marine fish larvae during the flexion, post-flexion and early juvenile stages (Margulies, 1993), along with an increase of growth hormone in the pituitary (Kaji et al., 1996, 1999a,b; Kaji, 2000) and digestive enzymes activity, displaying significantly different profiles among size classes when compared to other similarly-sized marine fish larvae (Buentello et al., 2011). The transition from a primitive digestive system at pre-flexion stage (4 DPH) to a differentiated, functional stomach upon post-flexion (16 DPH) resembling the adult type, might allow the tuna larvae to switch to a piscivorous feeding strategy earlier than other pelagic species, which in turn will bring rapid somatic growth. In exchange of this precocious development, tunas display an early point of no-return during the pre-flexion stage in which yolk reserves are
consumed very rapidly. The acute mortality corresponding to this period of exogenous feeding suggests poor nutrition as the main cause, as evidenced by degraded cellular condition in hepatocytes and intestinal epithelium cells (Margulies, 1993; Kaji, 2000).

In natural conditions, first-feeding BFT and YFT larvae feed mostly on copepods (Uotani et al., 1981), which have a high content of highly unsaturated fatty acids (HUFAS), essential for larval development and growth (Koven et al., 2018) (Tocher et al., 2008). The culture of copepods however, is challenging and variable, while harvests from the wild (e.g. plankton nets) is generally weather-dependent and labor-intensive (Margulies et al., 2016). As a result, rotifers and Artemia are commonly used as live feeds in the larval rearing of BFT and YFT, usually after an enrichment period with commercial products containing proteins, fatty acids, vitamins and minerals as these organisms have a lower nutritional value when compared to copepods (Katagiri et al., 2017; Margulies et al., 2001). Previous experiments on YFT larval rearing at the IATTC laboratory have been conducted over a 20-year period using feeding rates of 1-3 rotifers mL⁻¹ (3-14 DPH) and 0.1-0.25 Artemia mL⁻¹ (10-18 DPH) using AlgaMac 2000 or AlgaMac Enrich (Aquafauna BioMarine, Inc., Hawthorne, CA, USA) as enrichment media. At flexion stage, newly hatched YFT larvae are fed at 1-10 L⁻¹ (14-40 DPH), transitioning to minced fish upon complete metamorphosis (50-125% of body weight day⁻¹ from 25-100 DAH; (Margulies et al., 2016)). In other studies performed at this facility, Katagiri et al. (2017) used an enrichment mix of algae paste Nanno 3,600 (Reed Mariculture, Campbell, CA, USA) and Rotigreen Iso (Reed Mariculture), which included taurine as further supplementation, using a feeding rate of 10-28 rotifers mL⁻¹, from 2-14 DPH. Partridge et al. (2011) used Algamac 3050 (Aquafauna BioMarine, Inc.) as
enrichment media and fixed feeding rate of 5 rotifers mL\(^{-1}\) during 2-10 DPH and a stocking density of 15 YFT larvae L\(^{-1}\) (2 DPH).

In this study, Ori-One\(^{®}\) was used as enrichment media along with feeding rates of 3-10 rotifers mL\(^{-1}\) during 2-9 DPH. Enriched rotifers were further supplemented either with probiotics or 1-monoglycerides, assessing their effects on TL and survival of YFT larvae. Results from the probiotics trials showed a significantly greater TL in the probiotics group supplemented with the higher inclusion level (40\(\times\)10\(^6\) CFU L\(^{-1}\)) whereas no significant differences in TL were found in the supplementation at lower concentration (19\(\times\)10\(^6\) CFU L\(^{-1}\)). No significant differences in survival were found in either of the two probiotics. However, the mean survival at 10 DPH differed by a log order of magnitude in the two trials; in Trial 1, six larval rearing tanks stocked at 18 larvae L\(^{-1}\) produced 5,654 YFT larvae (7.5% overall survival) whereas in Trial 2, eight tanks stocked with twice as many larvae (40 L\(^{-1}\)) produced only 533 YFT (0.24% overall survival). Survival showed high variability among replicates in Trial 1, ranging from 0.3% to 13% in the control group, and from 1.1% to 18.8% in the supplementation group. In Trial 2, survival was much lower but less variable, ranging from 0.2% to 0.5% in control replicates and from 0.2% to 0.8% in supplemented replicates.

Margulies et al. (2016) have shown that growth of YFT is influenced by food availability and composition, rearing water temperatures, and stocking densities. They report that two-fold to four-fold increases in stocking density produce significantly slower growth rates in both, weight and length, in pre-flexion stage larvae, regardless of background food levels, while survival estimates for larvae and juveniles in density trials are not significantly different (Margulies et al., 2016). In the present study, TL was
significantly higher in the probiotics group supplemented with the higher dosage, which also had a higher and less variable survival rate compared to the control group. Under this scenario, probiotics might have assisted the rudimentary digestive system processes, enabling higher larval nutrient uptake and growth. The notable difference in overall survival found between the two probiotics trials may be explained by differences in stocking densities used in the experiments, and to detrimental effects associated with using a stocking density as high as 40 larvae L\(^{-1}\) in Trial 2. In this regard, Margulies et al. (2016) have investigated the effects of density (5, 10, 20, or 40 YFT larvae L\(^{-1}\)) on growth and survival at early stages. Their findings on survival showed that although the output was similar over the tested density range, any potential negative effects of high stocking densities in the yolksac stage occur at >40 yolksac larvae L\(^{-1}\).

With respect to the trial with 1-monoglycerides, TL at 9 DPH was higher in the supplementation group (4.26 ± 0.27 mm) compared to the control group (4.22 ± 0.21 mm) whereas DW was higher in the control (0.1086 ± 0.018 g) than in the supplemented group (0.0813 ± 0.014 g), with both of the mean differences not statistically significant. Even though no significant differences were found in DW, seven out of the eight experimental tanks showed an inverse relationship between DW and final larval abundance, as previously described by Margulies et al. (2016) on density-dependent studies on growth. No significant differences were found in survival; however, the supplementation group produced more than twice as many larvae (n=5,076) than the control group (n=2,472), which showed a particularly high variation (824 ± 949.5 larvae) compared to the supplemented group (1,692 ± 308 larvae); the survival percentage for both groups ranged from 0.9 to 8.5% (mean of 3.7%) and from 4.9 to 9.6% (mean of
7.5%), respectively. While survival was quite variable among replicates in each of the experiments carried out in this study, the survival obtained in the 1-monoglycerides-supplemented group yielded the higher and less variable results overall. We consider that the two-fold increase in the survival in the supplemented group, though not statistically significant, suggests a beneficial effect that might be related to additional energy sources and selective antibacterial action from the short-chain and medium-chain fatty acids provided through the 1-monoglycerides. The significance of such effects may have been masked by the combined effect of notable dispersion occurring in the control group and reduced sample size (n=3), leading to a type II error. This represents a common issue in YFT larval rearing experiments as sample size is generally limited by the availability of fertilized eggs and 1-2 DPH larvae; in this regard, taking into account that intermediate stocking densities (12-22 larvae L⁻¹) tend to yield higher survival, it is recommended that a balance between stocking density and number of replicates be considered for further trials, in order to increase the sample size for a better assessment of survival.

Growth and survival values obtained in this study are in accordance with those reported in other YFT studies and support previous findings on density dependent growth and potential negative effects of stocking larvae at >40 larvae L⁻¹ (Margulies et al., 2016; Katagiri et al., 2017; Partridge et al., 2011). Mortality rates for YFT at early developmental stages are considered high in nature and substantial under laboratory-rearing trials, with both periods, the yolk sac and first-feeding stages representing highest absolute mortality, reaching 80-95% mortality by 10 DPH in the laboratory (Lang et al., 1994; Margulies et al., 2016). The manipulation of physical conditions during YFT larval rearing has demonstrated improved performance in tuna at early developmental stages.
The IATTC group have conducted research for over 20 years including studies on the effects of microturbulence, light, water temperature, dissolved oxygen and pH on larval survival and growth, finding that subtle differences in these factors can produce surprisingly large differences in growth and survival (Margulies et al., 2016). Partridge et al. (2011) found that an extended photoperiod (24 hours of light at light intensities of 30 moles/m2/s during the night) in the larval rearing of YFT up to 9 DPH significantly increased both, survival and growth when compared to the control groups reared at 12 hours light: 12 hours dark. Survival of 9 DPH YFT larvae under this light regime conducted in duplicate trials ranged between $8.9 \pm 0.8\%$ to $10.4 \pm 1.9\%$ while control treatments ranged from $0.2 \pm 0.1\%$ to $1.0 \pm 0.3\%$. Growth and development were also significantly increased under extended photoperiod, whereas swim bladder inflation was significantly lower compared to the control. The significant increases in growth, survival and development were driven by an extended foraging time combined with the prevention of mortality caused by night time sinking. Similarly, in BFT extended photoperiod has increased survival and inhibited swim bladder inflation (Kurata et al., 2012) and also has shown increased survival in BFT juveniles (Honryo et al., 2013; Ishibashi et al., 2013). In other studies on physical conditions used in aquaculture trials, the addition of oil to the water surface resulted in significantly increased survival at 3 DPH and swim bladder inflation when compared to control groups (Honryo et al., 2016).

With respect to nutritional deficiencies in tuna species at early developmental stages, a few studies have addressed the effects of docosaheaxaenoic acid (DHA), choline and taurine supplementation on larval growth, mostly in BFT species, with only one study describing the taurine supplementation effects on growth performance and protein...
content of 14 DPH YFT larvae initially stocked at 12 larvae L$^{-1}$. In this study, the rotifer enrichment media (Nanno 3600 + Rotigreen Iso, Reed Mariculture) was supplemented with taurine (800 mg L$^{-1}$) using a feeding rate of 10-28 rotifers mL$^{-1}$ day$^{-1}$. Results showed that growth and total protein content were significantly increased in the supplemented group upon 8 DPH, with enhanced notochord development compared with the control, promoted by an increase in protein synthesis efficiency. Survival for taurine-supplemented and control group was 3.12 ± 1.21% and 3.33 ± 1.59%, respectively, with no statistically significant differences. Similar enhanced growth and protein ratios were also found in analogous experiments with BFT (Katagiri et al., 2017). Besides taurine, other compounds have been tested as food supplements for BFT larvae. Biswas et al. (2006) found significantly improved growth and survival feeding BFT larvae with Artemia sp. supplemented with docosaheaxaenoic acid (DHA) and choline, although performance was lower when compared to a reference diet of live fish larvae. Seoka et al. (2007), supplemented Artemia at different DHA inclusion levels for feeding 16 DPH BFT larvae (0.2-0.3 Artemia L$^{-1}$) stocked at 0.32 individuals L$^{-1}$. After 9 days of feeding, results showed a statistically higher survival in the supplement containing the mixing ratio of 1 DHA:0 oleic acid, whereas no significant differences in growth were detected. In a subsequent study, (Seoka et al., 2008) supplemented Artemia with salmon roe phospholipids, obtaining a similar response in growth and survival under phospholipids supplementation. In spite of the improvement of these variables, Biswas et al. (2006) and Seoka et al. (2007, 2008) point out that the performance under supplementation diets are still below the reference diets using live fish larvae. Research on different fields for improving growth and survival at early developmental stages of tunas has demonstrated
the effectiveness of feeds supplementation and modified environmental conditions (e.g. photoperiod, water surface, aeration, microturbulence, light intensity, feeding background contrast, water temperature, dissolved oxygen) that affect larval growth and survival. The present study found a significantly higher growth under high-concentration probiotics supplementation \( (40 \times 10^6 \text{ CFU L}^{-1}) \) and a two-fold increase (non significant) in survival under 1-monoglycerides supplementation compared to the control groups. Probiotics might have assisted digestive processes whereas 1-monoglycerides might have contributed as additional energy sources, both providing the YFT with a higher nutrient uptake and improved performance.

**CONCLUSION**

This study tested the effect of probiotics and 1-monoglycerides supplementation on the growth and survival of 9-10 DPH YFT larvae, an age commonly affected by poor nutrition and represented by acute mortalities. Results showed that the higher probiotic supplementation \( (40 \times 10^6 \text{ CFU L}^{-1}; \text{Trial 2}) \) yielded a significantly higher total length in the supplemented group compared to the control in 10 DPH YFT larvae. Supplementation with 1-monoglycerides yielded a two-fold increase in survival, which was not statistically different from the control group due to high variability (standard deviation). Probiotics might have assisted digestive processes whereas 1-monoglycerides might have contributed as additional energy sources and as selective antibacterial action, with both products providing the YFT with a higher nutrient uptake and improved performance. The results obtained here are in accordance with previous studies on YFT larval rearing, indicating some evidence of density dependence in growth and a potential
detrimental effect on survival due to higher stocking density $\geq 40$ larvae $L^{-1}$. Taking into account the high variability in survival among replicates in every treatment, a balance between stocking density and number of replicates is recommended to increase sample size and improve further survival assessments.


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