Aquaculture 526 (2020) 735395

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Autochthonous bacterium *Lactobacillus plantarum* as probiotic supplementation for productive performance and sanitary improvements on clownfish *Amphiprion ocellaris*

Peterson Emmanuel Guimarães Paixão[a], Márcia Valéria Silva do Couto[b], Natalino da Costa Sousa[b], Higo Andrade Abe[b], Ryuller Gama Abreu Reis[b], Joel Artur Rodrigues Dias[b], Juliana Oliveira Meneses[a], Fernanda Santos Cunha[a], Thays Brito Reis Santos[a], Izadora Cibely Alves da Silva[c], Estela dos Santos Medeiros[d], Rodrigo Yudi Fujimoto[e,*]

[a] Post Graduate Program in Health and Environment, Tiradentes University, Brazil
[b] Post Graduate Program in Animal Science, Federal University of Pará, Brazil
[c] Graduation in Biological Sciences, Tiradentes University, Brazil
[d] Graduation in Veterinary, College Pio Décimo, Brazil
[e] Brazilian Agriculture Research Corporation (Embrapa), Brazil

ARTICLE INFO

Keywords:
Clownfish
Captivity
Growth
Ornamental fish

ABSTRACT

This current study evaluated the probiotic dietary supplementation with autochthonous bacterium *Lactobacillus plantarum* for *Amphiprion ocellaris*. Therefore, it was performed an experiment in complete randomized design with four treatments (**T1:** Control without probiotic; **T2:** 10^4; **T3:** 10^6 and **T4:** 10^8 CFU/g of ration) and three replicates. Juvenile fish (n = 120; 0.46 ± 0.01 g and 0.26 ± 0.06 cm) were distributed in twelve aquarium (80 L) and it received trial meal three times a day at fed rate of 4% live weight during 90 days. The fish were monthly measured and determined the total length, standard length, height, weight gain, biomass gain, specific growth rate, apparently feed conversion, relative condition factor, uniformity and survival. At the end of experiment (90 days) fish were submitted to hematological (n = 9/ treatment; Erythrogram, Leukogram and Thrombogram) and histological analysis (n = 6/ treatment; Intestinal tract). Also, after the supplementation period, the fish were challenged with pathogenic bacteria and the survival and blood analyzed. Results showed the fish fed **T3** and **T4** had improvement on weight gain, feed conversion, specific growth rate, height and total height from the intestinal villi. Increases of red blood cell (Erythrocyte, hematocrit and hemoglobin) and white blood cell (Lymphocyte and monocyte) also observed to both treatments (**T3** and **T4**), However the T4 promoted the greater fish resistance to pathogenic bacteria. As conclusion, probiotic supplementation with the autochthonous bacterium *Lactobacillus plantarum* 10^6 CFU/g provides zootechnical improvement for clownfish rearing, but to improve the pathogenic infection resistance, the 10^8 CFU/g presented more adequate.

1. Introduction

The ornamental fish industry has grown around the world, having an estimated value of USD 100 billion in 2015 (Faria et al., 2016). In particular, ornamental marine fish farming holds valuable species in high regard (Rhyne et al., 2012; Murray et al., 2012; Ladisa et al., 2017). Currently, reef species remain the most reared ornamental fish in many countries, such as *Amphiprion ocellaris*, which is reared in ten different countries including Brazil (Fao, 2010, Rhyne et al., 2017).

Commonly named the anemonefish or clownfish, this reef species enjoys considerable popularity, given its vibrant colors and cinematographic history (Wabnitz et al., 2003; Doktor and Bui, 2018). Such fish adapt quickly to captivity due to their rapid and substantial reproduction (Kodama et al., 2011). Consequently, their production has increased across the world over time (Militz and Foale, 2017; Rhyne et al., 2017; Ladisa et al., 2017).

However, in order to facilitate intensive production, adequate nutrition is an essential factor to ensure health and welfare in captivity.

* Corresponding author.
E-mail address: rodrigo.fujimoto@embrapa.br (R.Y. Fujimoto).

https://doi.org/10.1016/j.aquaculture.2020.735395
Received 17 February 2020; Received in revised form 9 April 2020; Accepted 20 April 2020
Available online 21 April 2020
0044-8486/

Some immunological stimulants such as probiotic bacteria have been used to improve performance and health. Autochthonous acid lactic bacteria have been highlighted as providing specific benefits for the host (Sayes et al., 2017; Jatobá et al., 2018).

Autochthonous probiotics (such as *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Bacillus subtilis* or *Bacillus cereus*) increase weight gain and specific growth rate, improve feed conversion, and promote higher survival and beneficial immunological modulation with hematological changes and intestinal modulation in fish species (Nandi et al., 2017; Park et al., 2017; Jatoba et al., 2018; Liu et al., 2018; Dias et al., 2018).

Nonetheless, there remains little scientific literature regarding the use of allochthonous or autochthonous probiotic bacteria for rearing *Amphiprion ocellaris* (Wesseling et al., 2015). Due to this fish species' national and international commercial importance, new feed alternatives are necessary to provide better performance and increase resistance to infections. For these reasons, this study sought to evaluate autochthonous probiotic supplementation on the productive performance and immune modulation of juvenile clownfish (*Amphiprion ocellaris*) reared in captivity.

2. Material and methods

This study adhered to the ethical protocols for animal welfare CEUA number 010218. Isolated probiotics from six clownfish (*Amphiprion ocellaris*) underwent *in vitro* tests and were identified using the MALDI-TOFF method as *Lactobacillus plantarum* (Paixão et al., 2020). For the *in vivo* test, an experiment was conducted with a completely randomized design and four treatments (**T1**: Control without probiotic; **T2**: 10^4; **T3**: 10^6 and **T4**: 10^8 CFU/g of ration) and three replicates. The probiotic bacterium grown into Falcon tubes containing salinized Man Rugosa Sharped Broth (MRS Broth + NaCl 1.5%) incubated at 35 °C for 24 h. After bacterial growth, each Falcon tube containing the culture medium underwent serial dilution (factor 1:10) to determine the concentrations, and then sprinkled it at commercial ration (Tetra Marine Granules – crude protein 44%; lipid 11%; fiber 2%; minerals 9%; enriched with vitamin C and Omega-3 fat acid). Previously, the commercial diet was exposure to a UV radiation to inactivate any probiotic present. The *L plantarum* viability has a total of 15 days, but for this experiment, the diet was made each seven days.

A total of 120 clownfish (*Amphiprion ocellaris*) (mean weight 0.46 ± 0.01 g and 0.26 ± 0.06 cm) were acclimated in a glass aquarium (10 fish/aquarium 80 L) connected to a water recirculation system. The feed rate of 4% live weight and a feed frequency of three times a day (9 h, 12 h and 17 h) (Johnston et al., 2003) for 90 days were used. The water quality parameters remained stable, as follows: salinity (30.00 ± 0.48 ppt), temperature (28.07 ± 0.54 °C), pH (8.16 ± 0.06) dissolved oxygen (6.48 ± 0.36 mg/L), and total ammonia (0.29 ± 0.10 mg/L).

The fish were anesthetized monthly by immersion using benzocaine solution 20 mg/L (Pramod et al., 2010), and were then measured. The total length gain (final length – initial length), standard length gain (final standard length – initial standard length), height gain (final height – initial height), weight gain (final weight – initial weight), biomass gain (final biomass – initial biomass), specific growth rate (ln final weight – ln initial weight/ experiment days x 100), apparent feed conversion (apparent feeding intake / weight gain), relative condition factor (ln final weight/ b x ln initial length – a), uniformity (weight mean ± 20% / total fish x 100) and survival were determined.

At the end of the experiment (90 days), nine fish per treatment underwent blood evaluation. The blood was collected (1 mL) by caudal vein puncture using a sterile syringe with 3% EDTA. The number of erythrocytes (cell x 10^6 µL) was determined using a Neubauer chamber (Garcia-Navarro, 2005), hematocrit percentage was attained in accordance with Goldenfarb (1971), hemoglobin concentration ($mg.dL^{-1}$) was gauged with the aid of a biochemical analyzer (prestige IQ50), and total plasmatic protein ($g.dL^{-1}$) was measured using a refractometer (Quimis®). Following Vallada (1999), hematimetric indexes were calculated as mean corpuscular volume (MCV: Hematocrit x 100 / Erythrocyte fL), mean corpuscular hemoglobin (MCH: Hemoglobin x 10 / erythrocyte µg), and mean corpuscular hemoglobin concentration (MCHC: MCH x 100 / Hematocrit %). For leukocyte differential count, a blood smear was performed according Ranzani-Paiva (1995).

Subsequently, fragments of the intestinal tract (1 cm) from six clownfish per treatment received 10% formalin for 24 h until fixation and were then exchanged to 70% alcohol (Azevedo et al., 2016). The fragments underwent alcohol dehydration (70, 80, 90 and 100%) and then embedded into paraffin. Transverse cuts were made with 5 µm thick sections and stained by hematoxylin-eosin (HE). Histological cuts were analyzed by light microscopy, and measurements of intestinal morphometric parameters (total height, height, width and thickness) were taken in accordance with Silva et al. (2015).

As a final test, 45 clownfish were submitted to a trial challenge against pathogenic marine bacteria previously isolated from sick clownfish and identified as *Vibrio fluvialis via* the MALDI TOF method. To this end, an experiment was conducted with a completely randomized design, comprising five treatments and three replicates. Clownfish of each treatment from the probiotic supplementation (T1, T2, T3 and T4) received intraperitoneal injection with *Vibrio fluvialis* $1x10^5$CFU/mL and a negative control (T0), in which fish from T1 received sterile saline solution (Marudhupandi et al., 2017). The fish were kept in 10 L-tanks in a static system equipped with artificial aeration for 96 h. Every two (02) hours for four (04) days, clinical signs as well as mortality were determined (Ina-Salwany et al., 2019). Dead fish (during the trial time) and survivor fish at the end of 96 h were anesthetized and subjected to blood and microbiological analysis to determine Koch's postulates.

Normality and homoscedasticity tests (Shapiro-Wilk and Bartlett test, respectively) were applied to all data for productive performance and hematological evaluations, followed by analysis of variance (ANOVA) with the post-hoc Tukey test ($p < .05$).

3. Results

After 30 days, the fish showed higher weight gain, biomass and specific growth rate ($p < .05$) in treatment four (T4). The fish in treatment three (T3) presented similar feed conversion and standard length gain as those in treatment four (T4). All probiotic treatments improved height gain, although survival and relative condition factor did not differ statistically (Table 1).

After 60 days, treatments T2, T3 and T4 promoted a reduction in apparent feed conversion, and increase in length gain, weight and biomass gain compared to the control ($p < .05$). Furthermore, the fish in treatment four (04) exhibited the highest specific growth rate. No statistical difference between the treatments were observed to relative condition factor, survival and lot uniformity (Table 2).

At the end of the dietary supplemented experiment (90 days), treatment three (T3) promoted improvements in biomass gain, apparent feed conversion and specific growth rate. At this time, total length gain, standard length gain, total height gain, relative condition factor and survival did not differ between the treatments and the control (Table 3). Treatment four (T4) presented similarities in productive performance with treatment three (T3), apart from in terms of apparent feed conversion (AFC).

The lactic acid bacterium counted in intestines reflected the probiotic concentration added in ration (Table 4). Only treatment four (T4) manifested a low bacteria count in the intestine compared to the diet.

The probiotic supplementation in treatments three (T3) and four (T4) increased the intestinal villi (Table 5). Height and total height increases from treatment two (T2), although width and thickness increased from the treatment three (T3).

Table 1
Mean values ± standard deviation of productive performance from clownfish *Amphiprion ocellaris* submitted to probiotic supplementation during 30 days.

Parameters	T1	T2	T3	T4
WG(mg)	96.67 ± 29.63b	133.33 ± 21.25ab	144.00 ± 35.59ab	161.67 ± 17.44a
BG(mg)	967 ± 296b	1333 ± 212ab	1440 ± 356ab	1617 ± 174a
TLG(cm)	28.82 ± 0.49b	30.16 ± 0.34a	30.60 ± 0.57a	31.43 ± 0.89a
SLG(cm)	25.46 ± 0.31b	25.92 ± 0.30ab	27.93 ± 2.46a	27.30 ± 0.54a
THG(cm)	11.27 ± 0.31b	11.49 ± 0.22a	11.54 ± 0.21a	11.89 ± 0.10a
AFC	6.75 ± 1.77b	4.64 ± 0.74ab	4.60 ± 0.94a	3.76 ± 0.44a
SGR(%)	0.63 ± 0.17b	0.84 ± 0.12ab	0.90 ± 0.19ab	1.00 ± 0.10a
UNI(%)	80.00 ± 00.00a	56.67 ± 5.77b	80.0 ± 0.00a	56.67 ± 5.77b
Kr	1.00 ± 0.01a	1.00 ± 0.00a	1.00 ± 0.01a	1.00 ± 0.01a
S(%)	96.67 ± 5.77a	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a

WG-weight gain, BG- biomass gain, TLG-total length gain, SLG-Standard length gain, THG-Total height gain, APF-Apparent feed conversion, UNI-Uniformity, Kr-Relative condition factor, S-Survival, T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows mean statistical difference by Tukey test ($p < .05$).

Glucose values increased for all probiotic treatments (T2, T3 and T4). Furthermore, the fish in treatment four (04) showed higher total plasmatic protein also hematocrit and erythrocyte rates. Nonetheless, treatment three (03) presented a statistical difference for hematimetric index (MCV) and hemoglobin (Table 6).

In terms of white blood cells, treatment four (04) increased thrombocytes and neutrophils (Table 7). Similar results were found between treatment three (03) and four (04) with increased total leukocytes, but with reduction in lymphocytes. The treatment without probiotic supplementation (T1) had the lowest values for all blood parameters with the exception of basophils.

These blood changes also occurred after the experimental challenge, as reflected in the differences in survival rates. Treatment four (04) showed the greatest survival (100%) compared to the other treatments during the challenge (96 h) against *Vibrio fluvialis* 1 × 10^5 CFU/mL. The highest mortality rate (55%) occurred in the treatment without probiotic supplementation (T1) (Fig. 1).

The first clinical signs occurred in treatment one (01) after 24 h of the experimental challenge. Erratic swimming and cranial hyperemia represented the most frequent clinical signs until the end of the challenge (Table 8).

The clownfish in treatment one (01) exhibited a red spot on the head after 36 h, as well as epidermal depigmentation and liver hypertrophy (Fig. 2).

The clownfish in treatment one (01) after challenge showed increased glucose and lactate. Reductions in erythrocytes, thrombocytes, lymphocytes, basophils and neutrophils also occurred (Table 9).

4. Discussion

This is the first report into the use of autochthonous bacteria as probiotics for clownfish (*Amphiprion ocellaris*) reared in captivity. This bacterial supplementation is highly efficient in colonizing the intestinal tract and provides benefits compared to allochthonous bacteria (Jatobá et al., 2018; Hura et al., 2018). Dias et al. (2018) found that juvenile tambaqui (*Colossoma macropomum*) fed with the autochthonous bacterium *Bacillus cereus* for 120 days showed better productive performance. Moreover, the same results occurred with tilapia (*Oreochromis niloticus*) after 30 days of receiving autochthonous probiotic supplementation, showing increases in weight and specific growth rate (Gobi et al., 2018).

In the present study, intestinal modulation through bacterial colonization and changes in intestinal morphology explain the superior performance. The presence of probiotic bacteria in the intestinal tract during an unbroken feed period promotes the growth of villi and consequently greater nutrient absorption (Sayes et al., 2017; Munir et al., 2018). Thus, higher weight gain or better specific growth rate represent some of the benefits of autochthonous bacteria that have been reported in the literature and observed in clownfish. The opposite result occurred with goldfish (*Carassius auratus*) submitted to allochthonous probiotic *Lactobacillus helveticus*, as no improvement in productive performance was observed (Ahire et al., 2018).

The hematological profile provides important information about health conditions and nutritional aspects (Ranzani-paiva et al., 2013). The immunological system receives benefits from the probiotic supplementation during an unbroken period of feeding. Increased nutrient absorption thanks to greater numbers of intestinal villi result in a higher amount of glucose in the blood. This means that clownfish with probiotic diets have more energetic sources when compared to a control without probiotics (Gao et al., 2018, Abe et al., 2019). Dias et al. (2018) also reported this glucose alteration with a probiotic diet in tambaqui fish. Other changes in the blood, such as the increased hematocrit and hemoglobin observed in the present study, are in line with previous findings for carp (*Cyprinus carpio*) using the same species of

Table 2
Mean values ± standard deviation of productive performance from clownfish *Amphiprion ocellaris* submitted to probiotic supplementation during 60 days.

Parameters	T1	T2	T3	T4
WG(mg)	289.86 ± 77.45b	419.02 ± 25.02a	380.37 ± 38.59a	421.45 ± 22.78a
BG(mg)	2412 ± 1143b	3594 ± 330a	3803 ± 385a	3957 ± 473a
TLG(cm)	32.45 ± 0.48c	36.57 ± 0.39a	34.98 ± 0.30b	35.73 ± 0.49ab
SLG(cm)	28.44 ± 1.02b	31.41 ± 0.39a	31.17 ± 0.60a	31.75 ± 0.67a
THG(cm)	12.72 ± 0.57b	13.81 ± 0.30ab	13.18 ± 0.63ab	14.25 ± 0.55a
AFC	8.27 ± 1.83b	4.45 ± 0.30a	4.27 ± 0.29a	4.15 ± 0.38a
SGR(%)	1.58 ± 0.31b	2.14 ± 0.09ab	2.03 ± 0.13ab	2.15 ± 0.09a
UNI(%)	56.67 ± 20.82a	68.00 ± 19.08a	76.67 ± 20.82a	51.00 ± 33.81a
Kr	1.00 ± 0.01a	1.00 ± 0.00a	1.00 ± 0.01a	1.00 ± 0.01a
S(%)	93.33 ± 11.55a	93.33 ± 5.77a	100.0 ± 0.00a	96.67 ± 5.77a

WG-weight gain, BG- biomass gain, TLG-total length gain, SLG-Standard length gain, THG-Total height gain, APF-Apparent feed conversion, UNI-Uniformity, Kr-Relative condition factor, S-Survival. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows mean statistical difference by Tukey test ($p < .05$).

Table 3
Mean values ± standard deviation of productive performance from clownfish *Amphiprion ocellaris* submitted to probiotic supplementation during 90 days.

Parameters	T1	T2	T3	T4
WG(mg)	460.76 ± 23.74b	541.92 ± 23.59ab	564.86 ± 59.85a	563.56 ± 127.57a
BG(mg)	2180 ± 1141b	3101 ± 1425b	4604 ± 820a	3519 ± 838ab
TLG(cm)	37.24 ± 0.91a	36.87 ± 0.30a	36.94 ± 1.07a	37.32 ± 1.59a
SLG(cm)	31.76 ± 1.04a	31.45 ± 0.73a	31.37 ± 0.80a	31.26 ± 0.83a
THG(cm)	13.88 ± 0.10a	14.00 ± 0.17a	14.32 ± 0.31a	14.02 ± 1.01a
AFC	8.50 ± 3.52b	6.91 ± 2.91b	3.81 ± 0.53a	4.71 ± 0.94b
SGR(%)	2.14 ± 0.10b	2.58 ± 0.08ab	2.67 ± 0.17a	2.62 ± 0.41ab
UNI(%)	34.29 ± 10.17b	36.53 ± 15.90ab	58.13 ± 15.63ab	66.47 ± 24.10a
Kr	1.00 ± 0.01a	1.00 ± 0.01a	1.00 ± 0.01a	1.00 ± 0.01a
S(%)	73.33 ± 20.55a	76.67 ± 12.47a	90.0 ± 8.16a	80.0 ± 8.16a

WG-weight gain, BG- biomass gain, TLG-total length gain, SLG-Standard length gain, THG-Total height gain, APF-Apparent feed conversion, UNI-Uniformity, Kr-Relative condition factor, S-Survival. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows mean statistical difference by Tukey test ($p < .05$).

Table 4
Dietary and intestinal colonization with probiotic bacterium *Lactobacillus plantarum* after 90 days of experiment.

Local	T1	T2	T3	T4
Colonization in the diet (CFU/g)				
Ration	0.00 ± 0.00	2.64 ± 1.53 × 10^4	3.34 ± 1.15 × 10^6	2.33 ± 0.58 × 10^8
Colonization in the intestine (CFU/cm)				
L. plantarum	0.00 ± 0.00 × 10^0	2.33 ± 0.53 × 10^4	2.38 ± 0.78 × 10^6	1.34 ± 0.51 × 10^7
HB	5.36 ± 0.91 × 10^7	4.23 ± 0.10 × 10^7	1.58 ± 0.33 × 10^7	1.14 ± 0.45 × 10^7

Heterotrophic bacteria*. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL.

Table 5
Mean values ± standard deviation of morphometric villi from clownfish *Amphiprion ocellaris* submitted to the probiotic supplementation.

	T1	T2	T3	T4
T. Height (μm)	74.47 ± 1.28c	84.30 ± 2.69b	115.07 ± 4.67a	118.07 ± 4.53a
Height (μm)	53.10 ± 1.39c	60.43 ± 1.38b	91.47 ± 1.90a	89.17 ± 2.55a
Width (μm)	9.50 ± 0.65b	8.63 ± 0.38b	13.30 ± 0.79a	12.97 ± 1.01a
Thickness (μm)	5.22 ± 0.26b	5.42 ± 0.22b	6.93 ± 0.63a	7.73 ± 0.54a

T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows means statistical difference ($p < .05$).

Table 6
Mean values ± standard deviation of blood parameters from clownfish *Amphiprion ocellaris* submitted to the probiotic supplementation.

Parameters	T1	T2	T3	T4
Glu mg/dL	24.11 ± 5.11b	39.00 ± 2.60a	41.71 ± 2.81a	44.33 ± 8.03a
TPP (g/dL)	2.60 ± 1.30c	8.00 ± 2.00bc	10.73 ± 0.89b	10.07 ± 2.25a
Hema (%)	12.84 ± 0.95c	15.07 ± 0.81bc	16.81 ± 1.17ab	18.03 ± 0.65a
Eryth (x10^6)	1.00 ± 0.42b	1.69 ± 0.29ab	1.68 ± 0.54ab	2.00 ± 0.17a
Hemo (mg/dL)	6.42 ± 1.30b	8.00 ± 2.00ab	10.73 ± 0.79a	10.07 ± 2.25ab
MCV(fL)	102.76 ± 6.75ab	90.89 ± 11.66ab	107.40 ± 28.39a	87.83 ± 6.79b
MCH %	64.92 ± 28.27a	47.79 ± 20.66a	76.06 ± 23.00a	50.34 ± 12.55a
MCHC %	52.07 ± 13.13a	51.29 ± 14.60a	66.17 ± 4.52a	55.13 ± 11.88a

Glu-glucose, TPP-Total plasmatic protein, Hema-Hematocrit, Eryth-Erythrocyte, Hemo-Hemoglobin, MCV-Mean corpuscular volume, MCH-Mean corpuscular hematocrit, MCHC-Mean corpuscular hematocrit concentration. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows means statistical difference ($p < .05$).

Table 7
Mean values ± standard deviation for white blood cell of clownfish *Amphiprion ocellaris* submitted to probiotic supplementation during 90 days.

Parameters	T1	T2	T3	T4
Thrombocyte	1.11 ± 0.14b	1.10 ± 0.17b	1.20 ± 0.20b	2.71 ± 0.10a
Lymphocyte	16.90 ± 1.28a	16.50 ± 1.05ab	14.90 ± 1.21b	13.30 ± 1.33b
Monocyte	0.50 ± 0.08b	0.94 ± 0.19a	0.91 ± 0.24a	1.11 ± 0.25a
Neutrophil	2.01 ± 0.39c	2.02 ± 0.67c	3.20 ± 0.22b	4.95 ± 0.41a
Basophil	0.65 ± 0.05a	0.50 ± 0.04a	0.64 ± 0.07a	0.69 ± 0.17a
Total Leukocytes	34.07 ± 2.44ab	28.07 ± 1.06b	45.24 ± 5.98a	49.65 ± 7.09a

Thrombocyte cells/μLx10^3, Lymphocyte cells/μLx10^3, Monocyte cells/μLx10^3, Neutrophil cells/μLx10^3, Basophil cells/μLx10^3, Total Leukocytes cells/μLx10^4. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters in the rows means statistical difference ($p < .05$).

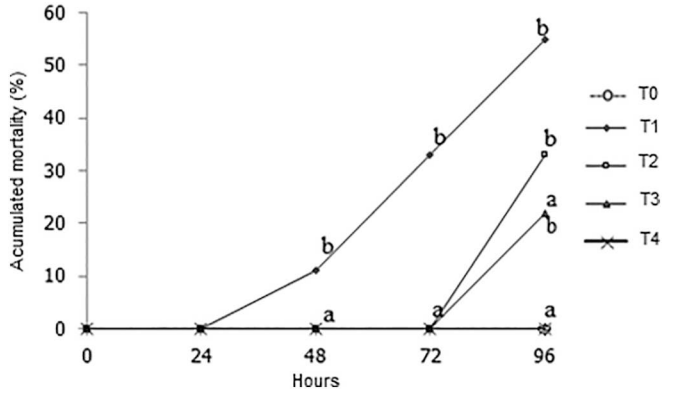

Fig. 1. Accumulated mortality during the challenge (96 h) against marine fish pathogen *Vibrio fluvialis*. T1-no probiotic, T2-1x10^{4}CFU/mL, T3-1x10^{6}CFU/mL, T4-1x10^{8}CFU/mL. Different letters means statistical difference in each observation time ($p < .05$).

Table 8
Clinical signs of clownfish *Amphiprion ocellaris* challenged by pathogenic bacterium *Vibrio fluvialis* during 96 h.

Clinical Signs	T0*	T1	T2	T3	T4
Erratic swimming	−	+	+	+	−
Epidermal depigmentation	−	+	+	−	−
Cephalic hemorrhage	−	+	−	−	−
Cranial hyperemia	−	+	+	+	−
Loss of balance	−	+	−	−	−
Accelerated opercula beat	−	+	−	−	−
Lethargy	−	+	−	−	−
Liver hypertrophy	−	+	+	−	−
Kidney hypertrophy	−	+	−	−	−
Liver opacity	−	+	−	−	−
Abdominal exudate	−	+	−	−	−
Internal hemorrhage	−	+	−	−	−

T0- Injection of sterile saline solution, (+) presence of clinical signs, (−) absence of clinical signs.

autochthonous probiotic bacterium *Lactobacillus plantarum* (Valiallahi et al., 2018).

Furthermore, defense cells like lymphocytes and neutrophils increased in this study, as also seen for *Labea rohita* using the probiotic bacterium *Bacillus* sp. (Nandi et al., 2017). According to Yamashita et al. (2017), neutrophils act as the first line of defense against various problems, including inflammation and bacterial infection. Increased neutrophils in the blood of clownfish (*Amphiprion ocellaris*) is an immune stimulant effect caused by probiotics.

Currently, resistance against bacterial infection related to probiotic supplementation has been widely reported in the literature. Tilapia (*Oreochromis niloticus*) submitted to a probiotic diet with *Bacillus licheniformis* showed immunological improvements, reflected in greater resistance to *Aeromonas hydrophila* (Gobi et al., 2018). Furthermore, dietary probiotic supplementation containing *Bacillus velezensis* in the feed of ornamental goldfish (*Carassius auratus*) can promote immunological resistance against the same kind of pathogenic bacterial infection (Yi et al., 2018).

Such resistance against pathogenic bacteria can be explained by the increase in leukocytes stimulated by the probiotic (Sayes et al., 2018, Jatobá et al., 2018; Dias et al., 2018). Liu et al. (2018) found that following probiotic supplementation, the ornamental striped beakfish (*Oplegnathus fasciatus*) exhibited greater phagocytic capacity against *Vibrio alginolyticus* due to higher numbers of leukocytes. A similar result was found in the present study with clownfish (*Amphiprion ocellaris*) receiving probiotic supplementation containing *Lactobacillus plantarum* 10^8 CFU/g, as they showed resistance against *Vibrio fluvialis* 10^5 CFU/mL.

Vibrio is the most common pathogen among marine ornamental fish around the world (Fioravanti and Florio, 2017; Lafferty et al., 2015). Indeed, the bacterium *Vibrio fluvialis* has already been reported for ornamental fish (Annie Selva Sonia and Lipton, 2012). Nonetheless, this is the first report regarding their infection in the ornamental fish *Amphiprion ocellaris*. This bacterium has a similar level of virulence as *Vibrio parahaemolyticus*, which infected sebae clownfish (*Amphiprion sebae*) at the same concentration of 10^5 CFU/mL (Marudhupandi et al., 2017).

Its clinical signs described in the literature (Huang and Wen-Wei Hsu, 2005; Annie Selva Sonia and Lipton, 2012), such as cephalic hemorrhage and cranial hyperemia, corroborate the present study during the trial challenge, and these clinical signs may explain the reduction in erythrocytes which was linked to hemorrhaging.

A reduction in leukocytes in fish without probiotic supplementation can be caused by the migration of cells to infection points. Nevertheless, fish that receive probiotics in their diets present greater numbers of lymphocytes against pathogenic bacterium after challenge. This elevated lymphocyte number in supplemented fish occurs due to the immunological improvement caused by probiotic supplementation, promoting more defense cells to combat the infection.

Despite the immunological improvement and greater resistance against pathogenic bacteria seen for clownfish submitted to the largest probiotic concentration (10^8 CFU/mL), productive performance remained similar to concentration of 10^6 CFU/mL. This difference between performance and immunological improvement might be connected to the physical limitations of the intestinal tract to the growth of probiotic bacteria. In an experiment using probiotic supplementation with striped beakfish (*Oplegnathus fasciatus*), the largest concentration (10^8 CFU/mL) also did not result in greater productive performance (Liu et al., 2018).

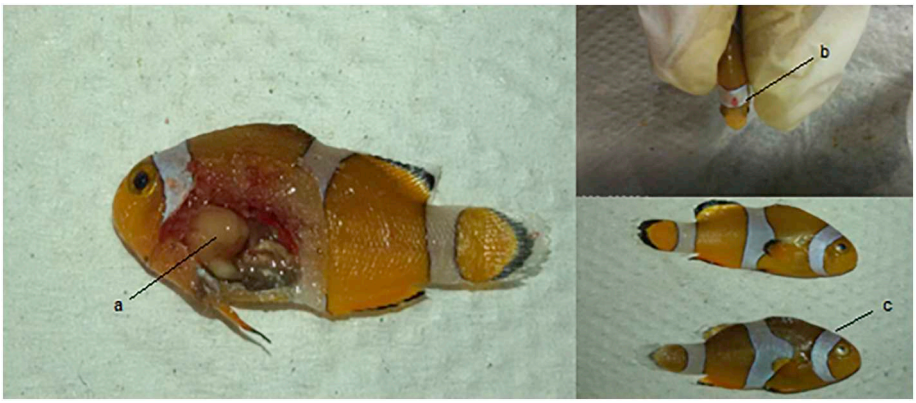

Fig. 2. Clinical signs of clownfish *Amphiprion ocellaris* challenged by pathogenic marine bacterium *Vibrio fluvialis* during 96 h. Liver hypertrophy (a), cephalic hemorrhage (b), epidermal depigmentation (c).

Table 9
Mean values ± standard deviation for blood parameters of the clownfish *Amphiprion ocellaris* after the challenge against *Vibrio fluvialis*.

	T0	T1	T2	T3	T4
Glucose	12.60 ± 2.41b	41.20 ± 16.36a	26.40 ± 7.16a	18.20 ± 6.34ab	18.00 ± 3.74ab
Lactate	0.90 ± 0.13c	2.28 ± 0.12a	1.43 ± 0.14b	0.98 ± 0.13c	0.90 ± 0.15c
Erythrocyte	0.82 ± 0.49ab	0.38 ± 0.23c	0.60 ± 0.22b	1.02 ± 0.49a	1.10 ± 0.35a
Thrombocyte	0.21 ± 0.01ab	0.05 ± 0.01b	0.18 ± 0.05ab	0.48 ± 0.02ab	0.80 ± 0.11a
Lymphocyte	2.15 ± 0.04ab	1.28 ± 0.02b	2.02 ± 0.13ab	3.67 ± 0.02a	3.95 ± 0.14a
Monocyte	0.20 ± 0.01ab	0.34 ± 0.02a	0.34 ± 0.01a	0.18 ± 0.02b	0.20 ± 0.02ab
Neutrophil	0.25 ± 0.04ab	0.09 ± 0.01b	0.22 ± 0.02ab	0.59 ± 0.04ab	1.01 ± 0.11a
Basophil	0.15 ± 0.02b	0.05 ± 0.01d	0.08 ± 0.01c	0.20 ± 0.01b	0.30 ± 0.04a
T.Leukocyt.	13.9 ± 0.48a	11.81 ± 0.12b	13.73 ± 0.29a	12.58 ± 0.12ab	13.16 ± 0.47ab

Glucose mg/dL, Lactate mg/dL, Erythrocyte cells/μLx10^6, Thrombocyte cells/μLx10^3, Lymphocyte cells/μLx10^3, Monocyte cells/μLx10^3, Neutrophil cells/μLx10^3, Basophil cells/μLx10^3, Total Leukocytes cells/μLx10^4. T1-no probiotic, T2-1x10^4CFU/mL, T3-1x10^6CFU/mL, T4-1x10^8CFU/mL. Different letters in the rows means statistical difference ($p < .05$).

5. Conclusion

The autochthonous bacterium *Lactobacillus plantarum* at concentration 10^6 CFU/mL provides greater productive performance. However, only the concentration 10^8 CFU/mL promoted higher resistance against pathogenic marine bacterium *Vibrio fluvialis* for clownfish *Amphiprion ocellaris*.

Statement of availability data

The data that support this study are availability with the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors have no conflict of interest to declare

Acknowledgement

The authors thank to National Council of Scientific and Technological Development (CNPq) by financial support to the Rodrigo Yudi Fujimoto (304533/2019-0)

References

Abe, H.A., Dias, J.A.R., Sousa, N.D.C., Couto, M.V.S.D., Reis, R.G.A., Paixão, P.E.G., Fujimoto, R.Y., 2019. Growth of Amazon ornamental fish Nannostomus beckfordi larvae (Steindachner, 1876) submitted to different stocking densities and feeding management in captivity conditions. Aquac. Res. 50 (8), 2276–2280.

Ahire, J.J., Mokashe, N.U., Chaudhari, B.L., 2018. Effect of dietary probiotic *Lactobacillus helveticus* on growth performance, antioxidant levels, and absorption of essential trace elements in goldfish (*Carassius auratus*). Probiot. Antimicrob. Proteins 1–10.

Annie Selva Sonia, G., Lipton, A.P., 2012. Pathogenicity and antibiotic susceptibility of Vibrio species isolated from the captive–reared tropical marine ornamental blue damsel fish, Pomacentrus caeruleus (Quoy and Gaimard, 1825). Indian J. Geo-Marine Sci. 41 (4), 348–354.

Azevedo, R.V.D., Fosse Filho, J.C., Pereira, S.L., Cardoso, L.D., Andrade, D.R.D., Vidal Júnior, M.V., 2016. Dietary mannan oligosaccharide and Bacillus subtilis in diets for Nile tilapia (Oreochromis niloticus). Acta Sci. Anim. Sci. 38 (4), 347–353.

Dias, J.A., Abe, H.A., Sousa, N.C., Couto, M.V.S., Cordeiro, C.A., Meneses, J.O., Carneiro, P.C., 2018. Dietary supplementation with autochthonous Bacillus cereus improves growth performance and survival in tambaqui Colossoma macropomum. Aquac. Res. 49 (9), 3063–3070.

Doktor, R., Bui, T., 2018. The symbiotic relationship of entrepreneurship and information technology. Int. J. Entrepre. Small Busin. 33 (4), 467–471.

Faria, P.M.C., Ribeiro, K., Almeida, C.F., Santos, F.W.M., Santos, R.F.B., 2016. Aquicultura ornamental: um mercado promissor. Panoram. Aquicult. 26, 24–37.

Fao. The ornamental fish trade: production and commerce of ornamental fish: technical-managerial and legislative aspects. 102, 2010, 0-134.

Fioravanti, M.L., Florio, D., 2017. Common diseases in marine ornamental fishes. Marine Ornament. Species Aquacult. 347–380.

Gao, X., Zhang, M., Li, X., Han, Y., Wu, F., Liu, Y., 2018. The effects of feeding *Lactobacillus pentosus* on growth, immunity, and disease resistance in *Haliotis discus* hannai Ino. Fish Shellfish Immunol. 78, 42–51.

Garcia-Navarro, C.E.K, 2005. Manual de Hematologia Veterinária. Carlos Eugênio Kantek, 2. pp. 206.

Gobi, N., Vaseeharan, B., Chen, J.C., Rekha, R., Vijayakumar, S., Anjugam, M., Iswarya, A., 2018. Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. Fish Shellfish Immunol. 74, 501–508.

Huang, K.C., Wen-Wei Hsu, R., 2005. Vibrio fluvialis hemorrhagic cellulitis and cerebritis. Clin. Infect. Dis. 40 (9), 75–77.

Hura, M.U.D., Zafar, T., Borana, K., Prasad, J.R., Iqbal, J., 2018. Effect of Commercial Probiotic *Bacillus megaterium* on Water Quality in Composite Culture of Major Carps.

Ina-Salwany, M.Y., Al-saari, N., Mohamad, A., Mursidi, F.A., Mohd-Aris, A., Amal, M.N.A., ... Zamri-Saad, M., 2019. Vibriosis in fish: a review on disease development and prevention. J. Aquat. Anim. Health 31 (1), 3–22.

Jatobá, A., Moraes, K.N., Rodrigues, E.F., Vieira, L.M., Pereira, M.O., 2018. Frequency in the supply of *Lactobacillus* influence its probiotic effect for yellow tail Lambari. Ciência Rural 48 (10).

Jatobá, A., Pereira, M.O., Vieira, L.M., Bitencourt, M., Rodrigues, E., Fachini, F.A., Moraes, A.V., 2018. Action time and feed frequency of Lactobacillus plantarum for Nile tilapia. Arq. Bras. Med. Vet. Zootec. 70 (1), 327–332.

Johnston, G., Kaiser, H., Hecht, T., Oellermann, L., 2003. Effect of ration size and feeding frequency on growth, size distribution and survival of juvenile clownfish, Amphiprion percula. J. Appl. Ichthyol 19 (1), 40–43.

Kodama, G., Annunciacao, W.F., Sanches, E.G., Gomes, C.H.D.A.M., Tsuzuki, M.Y., 2011. Viabilidade econômica do cultivo do peixe palhaço, Amphiprion ocellaris, em sistema de recirculação. Bol. Inst. Pesc. 37 (1), 61–72.

Ladisa, C., Bruni, M., Lovatelli, A., 2017. Overview of ornamental species aquaculture. FAO Aquacult. Newslett. 56, 39.

Lafferty, K.D., Harvell, C.D., Conrad, J.M., Friedman, C.S., Kent, M.L., Kuris, A.M., Saksida, S.M., 2015. Infectious diseases affect marine fisheries and aquaculture economics. Annual review of marine science 7, 471–496.

Liu, C.H., Wu, K., Chu, T.W., Wu, T.M., 2018. Dietary supplementation of probiotic, *Bacillus subtilis* E20, enhances the growth performance and disease resistance against *Vibrio alginolyticus* in parrot fish (*Oplegnathus fasciatus*). Aquac. Int. 26 (1), 63–74.

Marudhupandi, T., Kumar, T.T.A., Prakash, S., Balamurugan, J., Dhayanithi, N.B., 2017. Vibrio parahaemolyticus a causative bacterium for tail rot disease in ornamental fish. Amphiprion sebae. Aquac. Reports 8, 39–44.

Militz, T.A., Foale, S., 2017. The "Nemo Effect": perception and reality of Finding Nemo's impact on marine aquarium fisheries. Fish and Fisheries 18 (3), 596–606.

Munir, M.B., Marsh, T.L., Blaud, A., Hashim, R., Janti Anak Joshua, W., Mohd Nor, S.A., 2018. Analysing the effect of dietary prebiotics and probiotics on gut bacterial richness and diversity of Asian snakehead fingerlings using T-RFLP method. Aquac. Res. 49 (10), 3350–3361.

Murray, J.M., Watson, G.J., Giangrande, A., Licciano, M., Bentley, M.G., 2012. Managing the marine aquarium trade: revealing the data gaps using ornamental polychaetes. PloS one 7 (1).

Nandi, A., Banerjee, G., Dan, S.K., Ghosh, K., Ray, A.K., 2017. Probiotic efficiency of *Bacillus* sp. in Labeo rohita challenged by *Aeromonas hydrophila*: assessment of stress profile, haemato-biochemical parameters and immune responses. Aquac. Res. 48 (8), 4334–4345.

Paixão, P.E.G., Couto, M.V.S., Costa Sousa, N., Abe, H.A., Dias, J.A.R., Meneses, J.O., ... Yudi Fujimoto, R., 2020. In vitro selection of autochthonous lactic acid bacterium from clownfish Amphiprion ocellaris. Aquac. Res. 51 (2), 848–851.

Park, Y., Lee, S., Hong, J., Kim, D., Moniruzzaman, M., Bai, S.C., 2017. Use of probiotics to enhance growth, stimulate immunity and confer disease resistance to Aeromonas salmonicida in rainbow trout (Oncorhynchus mykiss). Aquac. Res. 48 (6), 2672–2682.

Pramod, P.K., Ramachandran, A., Sajeevan, T.P., Thampy, S., Pai, S.S., 2010. Comparative efficacy of MS-222 and benzocaine as anaesthetics under simulated transport conditions of a tropical ornamental fish Puntius filamentosus (Valenciennes). Aquac. Res. 41 (2), 309–314.

Ranzani-Paiva, M.J.T., 1995. Células do sangue periférico e contagem diferencial de leucócitos de tainha Mugil platanus Gunther, 1880 (Osteichthys, Mugilidae) da região estiarino-lagunar de cananéia-SP (LAT. 25º00'S–LONG. 47º55'W). Bol. Inst. pesc. 22 (1), 23–40.

Ranzani-paiva, M.J.T., Pádua, S.B., Tavares-Dias, M., Egami, M.I., 2013. Métodos para análise hematológica em peixes. Editora da Universidade Estadual de Maringá-EDUEM. pp. 9–115.

Rhyne, A.L., Tlusty, M.F., Schofield, P.J., Kaufman, L.E.S., Morris Jr, J.A., Bruckner, A.W., 2012. Revealing the appetite of the marine aquarium fish trade: the volume and biodiversity of fish imported into the United States. PLoS One 7 (5).
Rhyne, A.L., Tlusty, M.F., Szczebak, J.T., Holmberg, R.J., 2017. Expanding our understanding of the trade in marine aquarium animals. PeerJ 5.
Sayes, C., Leyton, Y., Riquelme, C., 2017. Probiotic bacteria as an healthy alternative for fish aquaculture. In: Savic, S. (Ed.), Antibiotics use in animals InTech Publishers, Rijeka, Croatia, pp. 115–132.
Silva, D.M., Valente, L.M.P., Sousa-Pinto, I., Pereira, R., Pires, M.A., Seixas, F., Rema, P., 2015. Evaluation of IMTA-produced seaweeds (Gracilaria, Porphyra, and Ulva) as dietary ingredients in Nile tilapia, Oreochromis niloticus L., juveniles. Effects on growth performance and gut histology. J. Appl. phycol 27 (4), 1671–1680.
Vallada, E.P., 1999. Manual de técnicas hematológicas-Livraria Atheneu. São Paulo, Rio de Janeiro, pp. 1–104.
Valiallahi, J., Pourabasali, M., Janalizadeh, E., Bucio, A., 2018. Use of *Lactobacillus* for improved growth and enhanced biochemical, hematological, and digestive enzyme activity in common carp at Mazandaran, Iran. N. Am. J. Aquac. 80 (2), 206–215.
Wabnitz, C., Taylor, M., Green, E., Razak, T., 2003. From ocean to aquarium: the global trade in marine ornamental species. UNEP/Earthprint 17, 6–64.
Wesseling, W., Wittka, S., Kroll, S., Soltmann, C., Kegler, P., Kunzmann, A., Lohmeyer, M., 2015. Functionalised ceramic spawning tiles with probiotic Pseudoalteromonas biofilms designed for clownfish aquaculture. Aquaculture 446, 57–66.
Yamashita, M.M., Pereira, S.A., Cardoso, L., Araujo, A.P., Oda, C.E., Schmidt, É.C., Mouriño, J.L.P., 2017. Probiotic dietary supplementation in Nile tilapia as prophylaxis against streptococcosis. Aquac. Nutr. 00, 1–9. https://doi.org/10.1111/anu.12498.
Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J., Wang, G., 2018. Probiotic potential of Bacillus velezensis JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. Fish Shellfish Immunol. 78, 322–330.