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# Innate immune response of pirarucu improved with yeast-supplemented diets

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ABSTRACT

The present study evaluated the effects of dietary yeast supplementation on the growth and blood parameters of *Arapaima gigas*. Fish were fed with commercial feed supplemented with NuPro® at 0, 10, 40 and 80 g / kg for 30 days and, did not exhibit any difference in growth. Blood was analyzed at the end of the feeding trial and after handling stress. A significant reduction in albumin and triglycerides levels was observed with increased supplementation of NuPro® in the diet of *A. gigas*. The interactive effect of diet and stress influenced plasma glucose levels, with a modulation at all levels of supplementation after stress, while in the control group the level was significantly high. NuPro® improved the innate immune response of *A. gigas* at all the tested concentrations, with a significantly higher levels number of thrombocytes, leukocytes, lymphocytes, monocytes, and neutrophils than the control group. There was also an increase in the number of thrombocytes after stress. As the inclusion of NuPro® in the diet improved the innate immune response at all the concentrations tested and effectively modulated plasma glucose levels, this product can be recommended as an immunostimulant for *A. gigas* fingerlings with the inclusion of 40 g/kg of feed.

## 1. Introduction

The increasing consumer demand for fish products has expanded fish farming to large-scale production, leading to changes in water quality and fish stress, as a consequence of handling, transportation, and high stocking densities. This stressful condition reduces fish immunity, increasing susceptibility to parasites and infectious diseases (Chakraborty and Hancz, 2011; Oliva-Teles, 2012). Diseases are one of main limiting factors of fish production, and can have a negative economic impact (Oliva-Teles, 2012; Biller-Takahashi and Urbinati, 2014). In Brazil, it has been estimated that disease causes approximately US$ 84 million in losses to national fish production (Tavares-Dias and Martins, 2017).

Effective management practices during fish farming would can reduce losses in production and productivity. Suitable nutrition is essential for maintaining the performance and health of farmed fish, thereby avoiding signs of nutritional deficiency (Oliva-Teles, 2012; Pohlenz and Gatlin III, 2014). Studies have shown that diet

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**Table 1**
Basal composition of commercial ration and proximate composition of experimental diets containing different concentrations of Nupro®.

| Parameters | Manufacturer's data | Diet (NuPro®/kg of feed) | | | |
|---|---|---|---|---|---|
| | | 0 g | 10 g | 40 g | 80 g |
| Dry matter (%) | 87 | 90.61 ± 0.07 | 90.12 ± 0.79 | 90.89 ± 0.15 | 90.97 ± 0.11 |
| Crude protein (%) | 45 | 48.55 ± 5.82 | 53.39 ± 0.65 | 53.61 ± 0.52 | 54.08 ± 0.36 |
| Ether extract (%) | 9 | 3.61 ± 0.56 | 3.38 ± 0.59 | 2.99 ± 0.59 | 3.55 ± 0.19 |
| Ashes (%) | 16 | 14.33 ± 0.10 | 14.07 ± 0.08 | 14.16 ± 0.17 | 13.03 ± 0.12 |
| Phosphorous (%) | 1 | – | – | – | – |
| Calcium (%) | 2 – 3 | – | – | – | – |
| Vitamin C (mg/kg) | 1500 | – | – | – | – |
| Vitamin E (mg/kg) | 400 | – | – | – | – |

Data expressed as mean values ± standard deviation.

supplementation can improve the growth and health of fish. Among such supplements, yeasts are exploited commercially in many production sectors (Souza et al., 2011; Pohlenz and Gatlin III, 2014). The most significant differentials favoring the use these microrganisms are the fact that they are rarely toxic or cause diseases (Souza et al., 2011), present high levels of protein, and are rich in B complex vitamins, especially thiamin, riboflavin, niacin, and pantothenic acid. Yeasts are therefore widely used in human and animal diets.

Yeasts are an important source of nucleotides. When added to diets, they contribute towards the reduction of stress and improving the health of the gastrointestinal system, modulating the immune system of fish (Trichet, 2010; Oliva-Teles, 2012). Many studies of the use of *Saccharomyces cerevisiae* in fish diets have shown that it lead to improvements in animal growth (Souza et al., 2013), hematological and immunological parameters and intestine morphology (Kafilzadeh et al., 2013; Pohlenz and Gatlin III, 2014). In recent studies, products containing *S. cerevisiae* extract have provided immunostimulatory effects on *A. gigas* fingerlings (Hoshino et al., 2017; Dias et al., 2019). However, considering the aquacultural importance of this fish species in the Amazon region, knowledge of topic remains limited. NuPro® is a yeast extract obtained through the removal of the cellular wall of a specific *S. cerevisiae* strain, and rich in nucleotides (which account for approximately 5% of its dry weight) (Berto et al., 2016). When used as a diet supplement, nucleotides can provide beneficial effects for the immune system, and for growth and cellular regeneration (Jarmołowicz et al., 2012, 2013; Berto et al., 2016). The mechanisms through which nucleotides stimulate fish immunity remain unclear (Jarmołowicz et al., 2012), but seem to be connected to high demand for the intense proliferation of nucleotides in the cells of the immune system. According to Jarmołowicz et al. (2012), immune cells are incapable of synthesizing new nucleotides in most tissues and depend on exogenous assimilation through the diet. Supplemental dietary nucleotides applied in anticipation of stressful events could ensure adequate circulation of the nucleotide pool, with beneficial effects for fish. Therefore, the present study aimed to evaluate the growth and hematological parameters of *Arapaima gigas* (pirarucu) fed with a yeast extract supplemented diet and dry brewery yeast (NuPro®) after an induced stress challenge.

## 2. Materials and methods

*A. gigas* fingerlings were acquired from a commercial fish farm (Rio Branco, state of Acre, Brazil) and were transported to the Aquaculture and Fisheries Laboratory, Embrapa Amapá (Macapá, state of Amapá, Brazil). They were acclimated to the laboratory conditions for 15 days in 1,000 L water tanks, with continuous aeration and water flow. Before the experimental phase, the fish were fed *ad libitum*, four times a day, with commercial extruded feed for carnivores containing 45 % crude protein (CP) (Nutripiscis AL 45E, Presence, Evialis do Brasil Nutrição Animal Ltda., Paulínia, São Paulo, Brazil). These experiments were carried out under authorization from the Ethics Committee for Animal Use of the Federal University of Acre, under procedural nr 23107009564/2014-29, and were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge - SisGen, under identification number A9FE48 F.

### 2.1. Experimental diets

Four treatments were used, one of which consisted of the commercial feed (control) only and three of which consisted of experimental diets that also included the use of NuPro® (Alltech Brazil). NuPro® is a supplement for animals comprising yeast extract and dry brewery yeast. According to the manufacturer's technical specification, the recommendation for use is that the supplement should account for 1–6% of the total diet for all animals, including fish. Thus, to determine which level would be ideal for pirarucu, NuPro® was incorporated into the commercial extruded feed for carnivorous fish at three concentration levels: 10 g of NuPro®/kg of feed; 40 g/kg of feed and 80 g/kg of feed. The group without the inclusion of NuPro® in feed was the control (denominated 0 g). To analyze centesimal composition of the experimental diets and NuPro®, 100 g of feed from each experimental diet was collected, in triplicate, according to the guidelines of the AOAC-Association of official analytical chemists (1995). Table 1 shows the chemical composition of the NuPro® and the experimental diets used for 30 days.

### 2.2. Experimental design

*A. gigas* fingerlings (n = 72) weighing 8.47 ± 0.32 g (mean values ± standard deviation) were distributed equally among 12 tanks of 100 L. Each treatment was conducted with three replicates of six fish each, giving a total of 18 fish per treatment. The fish were fed experimental diets four times a day (at 8:00; 10:00; 14:00, and 16:00 h), representing 10 % of their biomass, for 30 days.

On day 31, three fish were collected from each experimental unit for evaluation, totaling nine fish per treatment. Fish blood samples were collected and, biometry was performed immediately afterwards. The remaining fish were maintained in the experimental units and submitted to handling stress, which consisted of chasing the fish for five minutes using a net (Hoshiba et al., 2009), simulating the daily management activities of fish farms. Twenty-four hours after the fish had been subjected to stress to stimulate their immune system, blood samples were collected, and biometry was performed.

Water renewal and aeration within each tank were carried out continuously. The dissolved oxygen levels (6.52 ± 0.07 mg $L^{-1}$), temperature (29.1 ± 0.06 °C), and pH (5.66 ± 0.07) of the water were measured daily using a multiparametric probe (Hanna, USA, mod. HI 9829, Kyoto, Japan) and remained within the limits tolerated for maintaining the health of the fish.

### 2.3. Growth parameters

Initial weight (g) and final weight (g) data were used to calculate

the fish performance parameters:

*Weight gain* = *final weight- initial weight*

*Feed conversion ratio* (FCR) = *dry feed intake / weight gain*

*Daily growth index* (DGI) = 100 x (*final mean body weight*$^{1/3}$ - *initial mean body weight*$^{1/3}$) / *duration of trial*

*Voluntary feed intake* (VFI) = *crude feed intake* / (*initial mean body weight* + *final mean body weight* / 2) / *duration of trial*

*Hepatosomatic index* (HSI) = 100 x (*liver weight / body weight*)

*Viscerosomatic index* (VSI) = 100 x (*viscera weight / body weight*)

The fish body weight (g) and total length (cm) data were used to calculate the relative condition factor (Kn) according to the method recommended by Le Cren (1951).

### 2.4. Blood parameter analysis

The blood samples were collected by caudal vessel puncture, using needles and syringes containing sodium heparin. Each blood sample was used to determine the hematocrit (Ht) and hemoglobin concentration (Hb). These data were used to determine the mean corpuscular hemoglobin concentration (MCHC). The leukocyte respiratory activity (respiratory burst) was determined as described by Sahoo et al. (2005) and Biller-Takahashi et al. (2013), with absorbance readings from a spectrophotometer (Biospectro SP-220, Curitiba, Paraná, Brazil). Total leukocyte and thrombocyte counts and differential leukocyte counts were obtained using blood smears stained with May Grünwald-Giemsa-Wright (Ranzani-Paiva et al., 2013), by an indirect method (Ishikawa et al., 2008).

The remaining blood samples were centrifuged at 75 G for 5 min (Centrifuge 5424, Eppendorf, Hamburg, Germany) to obtain the plasma, in order to determine the levels of glucose, total protein, albumin, cholesterol, and triglyceride, using specific colorimetric kits (Labtest Diagnóstica S.A., Lagoa Santa, Minas Gerais, Brazil) for each metabolite, with absorbance readings from a spectrophotometer (Biospectro SP-220, Curitiba, Paraná, Brazil).

### 2.5. Statistical analysis

The data were firstly subjected to normality and homoscedasticity tests using the Shapiro-Wilk and Levene methods, respectively, and, where necessary, were transformed (mean corpuscular hemoglobin concentration, leukocytes respiratory activity, thrombocytes, leukocytes and lymphocytes number, plasma total protein and albumin) and analyzed using one-way and two-way ANOVA and post hoc Tukey test comparing means. Diet and stress were used as the main factors. Differences were considered significant at 5 % probability (Zar, 2010). The tests were performed using the Statistica 8.0 (StatSoft) software.

## 3. Results and discussion

The inclusion of 10, 40 and 80 g of NuPro® into the diet of *Arapaima gigas* diets for 30 days did not result in an increase in fish growth; however, no adverse alterations were observed and the fish displayed adequate growth for the species in this phase, when compared to the Control group (Table 2). This study agrees with that carried out by Hoshino et al. (2017) which also reported the absence of fish growth even after feeding with Mycosorb A + ®, a commercial yeast-containing product, for 45 days. Conversely, Dias et al. (2019) observed an improvement in pirarucu growth after feeding with a diet supplemented with 12 g Aquate Fish™/kg, a commercial feed additive containing *S. cerevisiae*, for 30 days. The studies above are pioneering in their use of these products in the diet of *A. gigas*, meaning that knowledge of their effects remains incomplete and further research is needed. The use of NuPro® in the diets of a variety of fish species has had mixed effects on the performance of the animals, as the results can be influenced by several factors, such as variations in the composition of the diet, the inclusion level of the product, the duration of the experiments, the amount of food provided, and the eating habits and size of the fish. Studies with *Ictalurus punctatus* (Peterson et al., 2012), *Sander lucioperca* (Jarmołowicz et al., 2012), and *Rachycentron canadum* (Lunger et al., 2006) did not identify differences in fish performance. Nevertheless, *Oreochromis niloticus*, *Onchorchyncus mykiss,* and *Dicentrarchus labrax* exhibited an improvement in growth performance when fed diets supplemented with NuPro® (Berto et al., 2016; Panagiotidou et al., 2009). It should also be considered that high levels of NuPro® may make the diet unpalatable by interfering with food intake and, consequently, negatively impact fish performance (Lunger et al., 2006; Peterson et al., 2012).

Hematological parameters provide important information on fish health and can be an indicator of animal welfare and suitable cultivation conditions. In the present study the inclusion of Nupro® did not cause alterations in hemoglobin concentration, hematocrit or burst parameters, when compared to the Control group (Table 3), which were within or above the means previously reported for fingerlings and juveniles of the pirarucu cultivated (Drumond et al., 2010; Hoshino et al., 2017; Dias et al., 2019) and similar to the findings of Hoshino et al. (2017). Similarly, no changes in leukocyte respiratory activity was found with the use of NuPro® in the present study, when compared to the Control group, and after handling stress. Dias et al. (2019) reported that pirarucu subjected to *Aeromonas hydrophila* infection exhibited an increase in hemoglobin and leukocyte respiratory activity when fed a 12 g Aquate Fish™/kg diet. It can be inferred that the different outcomes of the studies may be related to the mechanism that triggers leukocyte respiratory activity, enhanced by the presence of the pathogenic bacteria (Urbinati et al., 2015).

NuPro® (10, 40, and 80 g/kg of feed) in *A. gigas* diets caused increases in thrombocyte, total leukocyte, lymphocyte, neutrophil, and monocyte counts, thus demonstrating its effectiveness in improving the innate immunity of fish. Furthermore, the thrombocyte counts increased not only with NuPro® supplements, but also 24 h after stress management was applied. Nucleotides cause modulatory effects in the immune system cells, such as lymphocyte maturation and proliferation and increased in macrophage activity during phagocytosis (Berto et al., 2016). Also according to Berto et al. (2016), studies have confirmed that exogenous nucleotides may influence humoral or cellular immune responses in fish.

Thus, diets supplemented with nucleotides had an immunodulator effect on fish in the present study. This effect is extremely important to organism defense against stress and/or diseases during farming, as thrombocytes are responsible for blood coagulation and act in hormonal defense, performing a phagocytic function. This greater number is therefore essential for the maintenance of a healthy organism (Berto et al., 2016; Ranzani-Paiva et al., 2013). Leukocytes have an essential role in the nonspecific defense of the innate immune system of fish (Berto et al., 2016; Tavares-Dias et al., 2007). According to Drumond et al. (2010), lymphocyte are involved in immunoglobulin production, and modulate defense mechanisms, so that these leukocytes are considered the most important element of the immunological defenses of pirarucu. Neutrophils are the components of the immune system that form the first line of cellular defense against invaders and have a phagocytosis function. These cells proliferate in the circulation in response to infection, inflammation, and stress (Drumond et al., 2010). The principal phagocytes in fish are monocytes, as these cells have the capacity to migrate through the bloodstream to inflammation hotspots during infectious process (Ranzani-Paiva et al., 2013).

Two other studies have demonstrated that nucleotide-rich products, such as diet supplements, improve the immunological system of fish (Hoshino et al., 2017; Dias et al., 2019). The improvement of nonspecific immune parameters in fish fed diets supplemented with NuPro® has also been reported (Kowalska et al., 2015; Jarmołowicz et al., 2013; Berto et al., 2016). This immunomodulation is essential, given the fact that fish are more vulnerable during infection with opportunist pathogens. However, the nucleotide-stimulating mechanism in the

**Table 2**
Growth and body parameters of *Arapaima gigas* fingerlings fed with diets containing different concentrations of Nupro®.

| Parameters | Diet (NuPro®/kg of feed) | | | | *p* value |
|---|---|---|---|---|---|
| | 0 g | 10 g | 40 g | 80 g | |
| Initial weight (g) | 8.90 ± 0.78 | 8.61 ± 1.66 | 8.35 ± 1.90 | 8.02 ± 1.96 | ns |
| Final weight (g) | 52.03 ± 2.16 | 50.36 ± 5.43 | 51.55 ± 2.10 | 51.93 ± 3.96 | ns |
| Weight gain (g) | 43.13 ± 2.74 | 41.75 ± 4.72 | 43.20 ± 3.64 | 43.91 ± 4.20 | ns |
| Final length (g) | 21.54 ± 0.41 | 20.81 ± 0.67 | 21.43 ± 0.23 | 21.27 ± 0.80 | ns |
| FCR (g/g) | 0.76 ± 0.06 | 0.79 ± 0.09 | 0.76 ± 0.07 | 0.76 ± 0.09 | ns |
| DGI (%/Day) | 5.54 ± 0.33 | 5.48 ± 0.22 | 5.67 ± 0.61 | 5.78 ± 0.53 | ns |
| VFI (g/kg/Day) | 0.24 ± 0.01 | 0.25 ± 0.03 | 0.24 ± 0.01 | 0.24 ± 0.02 | ns |
| HSI (%) | 1.10 ± 0.10 | 1.01 ± 0.08 | 1.17 ± 0.16 | 1.16 ± 0.06 | ns |
| VSI (%) | 7.88 ± 0.31 | 7.62 ± 0.64 | 8.21 ± 0.39 | 7.94 ± 0.47 | ns |
| Kn | 0.99 ± 0.01 | 1.00 ± 0.01 | 1.00 ± 0.03 | 1.00 ± 0.01 | ns |

FCR: Feed conversion ratio, DGI: Daily growth index, VFI: Voluntary feed intake, HSI: Hepatosomatic index, VSI: Viscerosomatic index, Kn: Relative condition factor; ns: not significant.
Data expressed as mean values ± standard deviation (n = 18 per treatment).
Analysis by one-way ANOVA ($p > 0.05$).

**Table 3**
Blood and plasma parameters of *Arapaima gigas* fingerlings fed with diets with different concentrations of Nupro® and stressed by chasing.

| Parameters | Effects (P) | | | Factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet | Stress | Diet x Stress | Diet (NuPro®/kg of feed) | | | | Stress | |
| | | | | 0 g | 10 g | 40 g | 80 g | Before | After |
| Hemoglobin (g $dL^{-1}$) | ns | ns | ns | 12.75 ± 1.34 | 11.47 ± 2.14 | 12.72 ± 0.68 | 12.05 ± 0.88 | 12.01 ± 1.63 | 12.61 ± 0.75 |
| Hematocrit (%) | ns | ns | ns | 25.94 ± 3.00 | 25.30 ± 2.12 | 26.27 ± 1.11 | 25.62 ± 2.41 | 25.54 ± 2.79 | 26.02 ± 1.31 |
| Burst | ns | ns | ns | 0.31 ± 0.14 | 0.32 ± 0.07 | 0.34 ± 0.10 | 0.30 ± 0.05 | 0.32 ± 0.10 | 0.31 ± 0.09 |
| MCHC (g $dL^{-1}$) | 0.014 | ns | ns | 49.37 ± 1.74a | 44.80 ± 2.81b | 49.15 ± 2.85a | 49.70 ± 3.43a | 47.80 ± 3.82 | 48.71 ± 2.78 |
| Thrombocytes (x $10^3$ $\mu L^{-1}$) | < 0.01 | < 0.01 | ns | 5.91 ± 0.92c | 13.84 ± 7.58b | 23.02 ± 4.38a | 21.73 ± 4.82a | 13.54 ± 6.49 | 16.86 ± 9.32 |
| Leukocytes (x $10^3$ $\mu L^{-1}$) | < 0.01 | ns | ns | 34.43 ± 6.01c | 56.26 ± 10.47b | 92.14 ± 20.92a | 87.30 ± 7.63a | 70.31 ± 30.87 | 64.27 ± 24.23 |
| Lymphocytes (x $10^3$ $\mu L^{-1}$) | < 0.01 | ns | ns | 15.88 ± 2.84a | 26.58 ± 4.75a | 51.77 ± 10.61b | 50.36 ± 5.95b | 36.86 ± 17.66 | 36.28 ± 17.44 |
| Monocytes (x $10^3$ $\mu L^{-1}$) | < 0.01 | ns | ns | 2.60 ± 0.49c | 3.20 ± 0.97bc | 5.02 ± 1.35ba | 5.60 ± 0.96a | 4.07 ± 1.67 | 4.24 ± 1.57 |
| Neutrophils (x $10^3$ $\mu L^{-1}$) | < 0.01 | ns | ns | 14.53 ± 3.06b | 24.43 ± 4.35b | 39.28 ± 11.05a | 36.26 ± 4.32a | 29.44 ± 13.45 | 27.51 ± 10.73 |
| PAS-LG (x $10^3$ $\mu L^{-1}$) | ns | ns | ns | 0.57 ± 0.26 | 0.39 ± 0.17 | 0.36 ± 0.39 | 0.54 ± 0.35 | 0.49 ± 0.28 | 0.46 ± 0.33 |
| Eosinophils (x $10^3$ $\mu L^{-1}$) | ns | ns | ns | 0.84 ± 0.27 | 0.89 ± 0.37 | 0.73 ± 0.28 | 0.45 ± 0.43 | 0.73 ± 0.27 | 0.69 ± 0.47 |
| Total Protein (g $dL^{-1}$) | ns | ns | ns | 2.34 ± 0.37 | 2.31 ± 0.36 | 2.19 ± 0.47 | 1.71 ± 0.17 | 2.12 ± 0.46 | 2.12 ± 0.41 |
| Albumin (g $dL^{-1}$) | 0.017 | ns | ns | 0.66 ± 0.22a | 0.41 ± 0.06b | 0.34 ± 0.04b | 0.40 ± 0.08b | 0.44 ± 0.17 | 0.48 ± 0.19 |
| Cholesterol (mg $dL^{-1}$) | 0.004 | ns | ns | 120.56 ± 11.45a | 134.38 ± 18.03ab | 158.07 ± 16.67b | 157.83 ± 17.62b | 140.49 ± 22.56 | 144.93 ± 22.67 |
| Triglycerides (mg $dL^{-1}$) | < 0.01 | ns | ns | 98.42 ± 16.18a | 108.62 ± 21.29a | 88.66 ± 20.16ab | 67.58 ± 10.66b | 98.31 ± 27.31 | 83.33 ± 13.96 |

MCHC: mean corpuscular hemoglobin concentration; Burst: Leukocytes respiratory activity; PAS-LG: Granular leukocyte PAS positive; ns: not significant.
Fish blood samples were collected before (n = 9 per treatment) and 24 h after handling stress (n = 9 per treatment).
Data expressed as mean values ± standarddeviation. Different letters = significant differences in two-way ANOVA, analysis followed by post-hoc Tukey ($p < 0.05$).

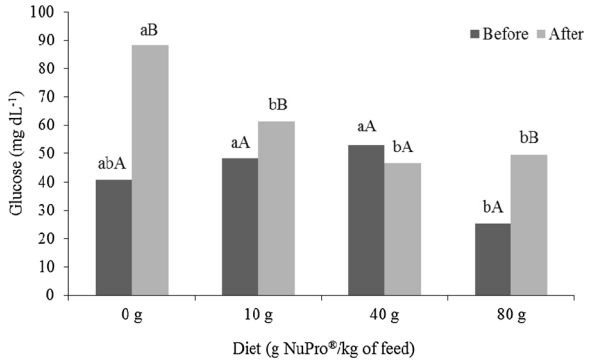


**Fig. 1.** Effects of diet and stress interaction (mean) on plasma glucose concentration of *Arapaima gigas* fingerlings fed diets with different Nupro® inclusion levels. Fish blood were sampled before (n = 9 per treatment) and 24 h after handling stress (n = 9 per treatment). Different uppercase letters indicate significant differences ($p < 0.05$) in each levels of Nupro® inclusion, before and after stress. Lowercase letters indicate significant differences ($p < 0.05$) between diets before or after stress.

immune activity of fish has not been fully explained. According to Jarmołowicz et al. (2012), it is most likely linked to the increased demand for cells in the immune system due to the intense proliferation of nucleotides. A combination of aquaculture practices in commercial production and stress factors can negatively impact the resistance of fish, causing disease outbreaks and mass mortalities (Kowalska et al., 2015), demonstrating the importance of maintaining the immune system of the fish at suitable levels.

The reduction of plasma albumin levels was observed at all the levels of NuPro® inclusion evaluated in the present study. In addition, *A. gigas* fed with 40 and 80 g / kg of NuPro® showed triglyceride reduction and increased plasma total cholesterol. The supplementation of nucleotide-rich products in fish diets produced a variety of effects on plasma parameters, which could be related to factors such as feeding trial time duration, level of inclusion of product, and others. As an example, Kowalska et al. (2015) exhibited total cholesterol, albumin and triglycerides levels reduction in *S. lucioperca*; Dias et al. (2019) reported heightened levels in plasma triglycerides and total cholesterol in *A. gigas* and, Hoshino et al. (2017) reported plasma triglyceride reduction in *A. gigas* with Mycosorb A+® diet supplementation, and plasma total cholesterol increasing at the highest evaluated level of inclusion.

An interactive effect between diet and stress was observed in relation to glucose concentration (Fig. 1), demonstrating that the negative effect of stress on fish health depends on the dietary inclusion of NuPro®. This regulated the post-stress glucose levels in all the groups receiving the supplement. In the non-supplemented (control) group, the glucose level was significantly higher in fish subjected to handling stress. This interaction between diet and stress enabled better regulation of glycemic levels when NuPro® was added to the diets, compared with the control group. Glucose concentrations are related to a series of stress agents that include temperature variation, handling, and transportation (Urbinati et al., 2015). Thus, glucose has been used as a good secondary indicator of stress in fish (Brandão et al., 2006), as an increase in plasma glucose levels occurs after the immediate increase of cortisol levels. Recovery to baseline glucose levels typically occurs 24 h after stress, and these were observed in the present study in fish fed with diets containing NuPro®, indicating that supplementation contributed towards modulation of the glucose levels of *A. gigas*.

## 4. Conclusion

The inclusion of NuPro® in the diet of *A. gigas* provided nonspecific immune response improvements at all concentrations evaluated. Thrombocyte counts after stress also increased, indicating that this may constitute a defense reaction. NuPro® was also found to be an effective modulator of plasma glucose levels, given the degree of recovery observed among the fish that received the supplement. The inclusion of 40 g of NuPro®/kg of feed is recommended as the best concentration to offer this fish species at the beginning of its development.

## Declaration of Competing Interest

This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication and is not under consideration for publication elsewhere. This research has not been submitted for publication nor has it been published in whole or in part elsewhere. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to Aquaculture Reports.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100421.

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