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# Assessment of the oxidative state, related parameters and quality of muscle tissue in Nile tilapia with the application of homeopathic product Homeopatila 100® in high-density cages

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**The impact using Homeopatila 100® on cultured Nile tilapia in high-density cages (1240 individuals) was assessed by measuring the oxidative state and related parameters as well as the quality of muscle tissue. Males with sexual inversion from a homogenous tilapia population were randomly distributed in 10 cages with storage volume of units of 1.2 $m^{-3}$ for a period of 91 days. Two diets were assessed: 1 (control) to 40 ml of hydroalcoholic solution (alcohol 30° GL)/kg of feed; 2 to 40 ml of the homeopathic product per kg of feed. The experiment involved the monitoring of physical and chemical parameters of water. At the final stage of the experimental period, hepatoprotective capacity of the homeopathic product Homeopatila 100® was assessed. This was done by the analyses of carbonyl protein, GSH and antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx)] in livers of Nile tilapia as well as the serum concentrations of biomarkers of hepatic damage (aspartato transaminase (AST), alanina transaminase (ALT) and FAL). In order to assess the quality of the fish' muscle tissue, the values of pH, color, texture and water retention capacity was measured. No statistical difference was observed regarding the physical and chemical parameters of water nor the analyses of the oxidative state and related parameters between treatments. Water retention capacity and texture were significantly higher ($p<0.05$) for the control treatment, while luminosity was higher for treatment with Homeopatila 100® ($p<0.05$). Nile tilapia cultivated in high density that received the homeopathic product Homeopatila 100® incorporated into their feed presented better-quality muscle tissues when compared with the group control. Homeopatila 100® did not indicate modifying effect on the oxidative state of Nile tilapia tissue cultivated in high density.**

**Key words:** Tilapia cultivation, oxidative state, enzymes, homeopathy, stress, muscle tissue.

## INTRODUCTION

Increase in fish consumption over the past few years has provided Brazil with an enormous potential for the development of fisheries and aquaculture enabling the country to become a future major fish producer worldwide. According to a Ministry of Fisheries and quaculture (MPA) Survey (2013), the country produces

approximately 2.5 million tons of fish with a population consumption of 17.3 kg per capita/year, near the world average released by the World Health Organization (WHO, 2012).

Nile tilapia is one of the most important fish species in cultivation in the country with production increasing on average 17% a year (MPA, 2013). The characteristics of tilapia are extremely favorable for cultivation (fast development, tolerance to a great variety of environmental conditions, capacity to reproduce in captivity, resistance to stress and diseases) (El-Sayed, 2006). Tilapia have meat of good acceptance in the market, good organoleptic characteristics and great industrial and culinary versatility (Furuya, 2010).

Simultaneously, with the increasing fish production, rearing animals has evolved from an artisanal to an industrial system in the world (Food and Agriculture Organization of the United Nations [FAO], 2014). The search for more profitable has led to intensive production, with the animals submitted to more stress. This has reduced the fishs' defense capacity with negative reflection on productivity and increasing occurrences of disease (Real, 2008).

To improve the production and quality of fish, homeopathy has been seen as an alternative management approach. The application of homeopathy to the herds consists in the Population Homeopathy, developed to attenuate the stressful model of animal production and assure minimum welfare to animals (Real, 2008). Several studies have demonstrated excellent results with the use of homeopathy in Nile tilapia (Andretto et al., 2014; Braccini et al., 2013; Merlini et al., 2014; Piau et al., 2012; Siena et al., 2010; Valentim-Zabott, 2008).

Fish are known for their high nutritional value (Godoy et al., 2010) and a quality muscle tissue (fillet) that can be measured through parameters such as pH, color, water retention capacity and softness, all features that can be influenced by animal stress (Koblitz, 2008).

The objective of this study was to assess the effectiveness of Homeopatila 100® on improved quality of muscle tissue of Nile tilapia in high-density cages by testing the oxidative state and related parameters.

## MATERIALS AND METHODS

The experiment was approved by the Animal Experimentation Ethics Committee of the State University of Maringá with approval under Protocol 092/2013 (Annex 1).

### Location and period

The experiment was conducted in Corvo River, Diamante do Norte, Paraná State (Figure 1). The 91-day experiment started in March 2014.

### Physical and chemical parameters of water

The mean values of the physical and chemical parameters of water such as temperature, pH and dissolved oxygen were recorded once a week and assessed twice a day for 9 am and 4 pm at seven different points near the cages (Figure 2). Temperature and oxygen were monitored with oximeter model YSI-55/12 FT (Aquatic Eco-Systems®).

### Fish, installations and feeding

Males with sexual inversion belonging to a homogenous population of Nile tilapia with mean initial weight of 50.24±8.51 g in the control treatment and 50.05±8.56 g in treatment with Homeopatila 100® were randomly distributed in 10 cages with a volume of 1.2 $m^3$ (1.0 × 1.0 × 1.2 m tall).

124 fish per cage were distributed. Before the beginning of the experiment, the fish were acclimated for seven days in cages, to adapt to the quality of water, density, food and management. Two treatments were assessed with five repetitions each through a fully randomized experimental design numbering 620 fish per treatment. The animals were manually fed with extruded commercial feed (5 mm) containing 32% crude protein three times a day (8:00 am, 1:00 pm and 5:00 pm) according to the recommended quantities for the species based on fish weight. The bromatological analysis of the feed was carried out at the Laboratory of Food Analysis of the State University of Western Paraná.

### Treatments

Based on the results of Siena et al. (2010) who had shown the effect of Homeopatila 100® on Nile tilapia male fries with sexual inversion with better results in fish that received 40 $mlkg^{-1}$ of feed, established two treatments: control (40 ml hydroalcoholic solution per kg of feed) and *Homepatila 100* ®(40 ml per kg of feed).

Product Homeopatila 100® in the form of hydroalcoholic solution was incorporated into the feed directly using mechanically-moved sprinkler irrigation on a weekly basis, homogenized and air-dried for 24 h protected from direct sunlight. The same process was conducted for the control treatment. The complete feed was acclimated in a ventilated area without sunlight, chemical products or equipment emitting magnetic field until loose and without alcohol odor. Homeopatila 100® was developed by REAL H, a company in Campo Grande (MS); its official composition is as shown in Table 1. The product is registered in the Ministry of Agriculture, Livestock and Food Supply.

### Assessment of oxidative state and related parameters

The assessment of oxidative state and related parameters was carried out at the laboratory of Hepatic Metabolism of the State University of Maringá. At the final stage of the experiment, 3 fish from each cage (15 fish per treatment/3 per cage) were anaesthetized with Benzocaine(dosage of 1 g/10 L of water) according to Stoskopf (1993). Firstly, blood sampling from Nile

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**Figure 1.** Cages in Corvo River, Diamante do Norte, Paraná.

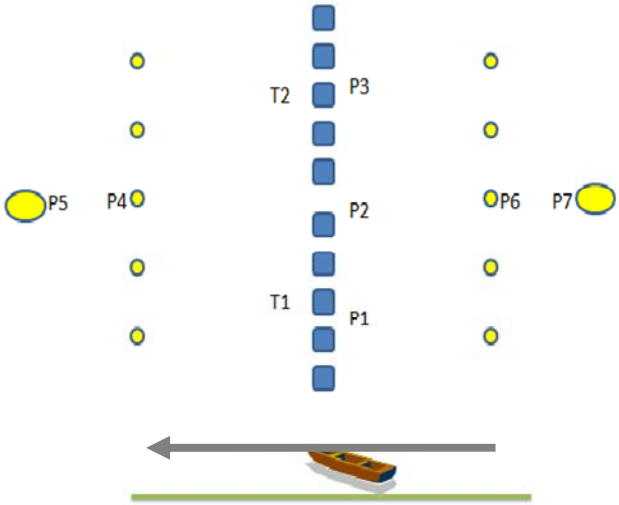


**Figure 2.** Monitoring points of physical, chemical parameters of water. The direction of the water flow is indicated with the arrow (right to left).

tilapia was conducted to perform enzymatic testing. On immobilization, total blood was collected through tail vase puncture, without the presence of anticoagulant to obtain serum. The serum was obtained after 3000 rpm centrifugation for 15 min. The serum was used to assess the enzymatic activities glutamate-oxaloacetate transaminase (AST), glutamate-pyruvate transaminase (ALT) and alkaline phosphatase (FAL). This was conducted with commercial kits (Gold Analisa diagnostica LTDA).

The livers were then collected after euthanasia conducted through severing the spinal cord. The abdominal cavity was surgically exposed; livers of Nile tilapia were removed, clamped in liquid nitrogen and stored at temperatures <-80°C. In order to prepare the homogenate, the clamped liver was weighted (1 g) and homogenized in Van Potter Elvehjem homogenizer with seven volumes of potassium phosphate buffer 0.1 M (pH = 7.4). The liver homogenate was used to analyze dosage of proteins and reduced glutathione (GSH). To establish the enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), dosage of protein and carbonyl protein, the homogenate was centrifuged at 10000 rpm for 15 min

**Table 1.** Composition of Homeopatila 100®.

| Compound | Dilution |
|---|---|
| Iodum | 12 CH |
| Sulphur | 30 CH |
| Natrum muriaticum | 200 CH |
| Streptococcinum | 30 CH |
| Vehicle (Ethyl alcohol 30° GL) | Q.s.p. |

Q.s.p.: Quantity sufficient for. Source: REALH – Campo Grande – MS – Brazil

using supernatant.

#### Protein dosage

The methodology of Lowry et al. (1951) was used on the total homogenate and centrifugation supernatant.

#### Establishing the level of reduced glutathione (GSH)

The level of reduced GSH was established through spectro-fluorimetry according to the description by Hissin and Hilf (1976) with alterations (Pardo-Andreu et al., 2007). The concentration of reduced glutathione was expressed as µg of GSH per mg of protein.

#### Carbonyl protein

The content of carbonyl proteins (CO) was established using the method of 2.4 dinitrophenylhydrazine (DNPH) as described by Levine et al. (1990). The content of carbonyl groupings was calculated based on the molar extinction coefficient of 22 $mM^{-1}/cm^{-1}$, and the results expressed in nmoles of carbonyl groupings per mg of protein.

#### Establishing catalase activity (CAT)

The activity of the catalase enzyme was assessed through enzymatic decomposition of $H_2O_2$ directly measured through spectrophotometry in 240 nm, according to Aebi (1974). The material used to establish the catalase was the supernatant obtained through the centrifugation of the liver homogenate. The activity of the enzyme was calculated using the molar extinction coefficient obtained from a calibration curve with $H_2O_2$, and the values expressed as µmols of $H_2O_2$ per min/mg of protein in the supernatant.

#### Establishing superoxide dismutase (SOD) activity

The activity of enzyme superoxide dismutase (SOD) was established through its capacity to inhibit the self-oxidation of pyrogallol reagent in alkaline medium, which can be monitored through spectophotometry in 420 nm (Marklund and Marklund, 1974). The activity in the homogenate supernatant was expressed as U of SOD per mg of protein in the supernatant.

#### Establishing GR activity

The activity of the GR enzyme was established through the decrease in absorbance due to the consumption of NADPH in 340 nm (Bergmeyer et al., 1974). The activity in the supernatant of the liver homogenate was expressed as µmol of NADPH per min/mg of supernatant protein.

#### Establishing glutathione peroxidase (GPx) activity

The activity of the GPx enzyme was established through the decrease in absorbance due to the decomposition of NADPH dependent on $H_2O_2$ in 340 nm at 25°C (Paglia and Valentine, 1967; Tappel, 1978). The activity in the supernatant of the homogenate was expressed in nmol of the NADPH/min/mg of supernatant protein.

#### Assessment of muscle tissue in Nile tilapia

To assess the quality of muscle tissue in the fish of both treatments, the analyses of pH, color, texture and water retention capacity were carried out at the Laboratory of Meats of the Federal Technological University of Paraná, Medianeira Campus, PR, Brazil.

All equipment and implements were sanitized with sodium hypochlorite solution (150 ppm) for 15 min prior to usage. The fish (15 fish per treatment/3 per cage) were gutted, had fins and skins removed and were filleted. The fillets were washed in chlorinated water (5 ppm) and immediately placed in polyethylene bags and stored (-18°C) until the analyses.

The measurement of pH was conducted on the fillet at room temperature using potentiometer (pH 21, Hanna®, Romania). Color was measured using colorimeter (Model CR 400, Minolta®, Japan) with illuminant D65 and viewing angle of 10° in three different surface points of the fillet, corresponding to the central and lateral part of the samples (MacDougall, 1994; Perlo et al., 2006). The values of $L^*$ (Luminosity), $a^*$ (red-green component) and $b^*$ (yellow-blue component) were expressed according to CIELAB color system (Minolta, 1998).

To assess texture (softness) through shearing force, the fillet was cut into 5 pieces of 1.5 cm tall × 1.0 cm wide x 2 cm long. The analyses were conducted with texturometer (TA.HD plus, Stable Micro Systems, UK) equipped with Warner-Brazler blade with a load cell of 5 kg. The blade was operated at a speed of 5.0 $mms^{-1}$ for a distance of 20 mm (± 0.001 mm). The results of the minimum force required to perform the section were expressed in Newton (N).

The water retention capacity was measured as per Hamm (1960). The determination was based on the measure of the released water lost when applied pressure on the muscle tissue. Meat cubes (2 g) were inserted in filter papers and placed between two glass plates with a weight of 10 kg applied for 5 min. After the pressure, the fillet sample was weighted, and the difference in weight reflecting the quantity of lost water. The result was expressed in percentage of exudate water in relation to the initial weight of the sample.

#### Statistical analysis

To verify the existence of differences in the values of water parameters, Kruskal-Wallis H test ($p \leq 0.05$) (Ayres et al., 2000) was applied. Oxidative state and parameters, as well as the quality of muscle tissue were analyzed by Student's t-test, using GraphPad Prism® software.

## RESULTS

### Physical and chemical parameters of water

There was no significant difference in water temperature,

**Table 2.** Mean values of water parameters during the experiment.

| Observed parameter | Temperature (°C) | pH | Dissolved oxygen (mg/L ) |
|---|---|---|---|
| P 1 | 25.35±3.32[a] | 8.82±0.23[a] | 9.98±2.57[a] |
| P 2 | 25.80±4.10[a] | 8.24±0.09[a] | 9.28±2.22[a] |
| P 3 | 25.85±4.17[a] | 8.15±0.12[a] | 8.95±2.26[a] |
| P 4 | 25.90±4.10[a] | 8.02±0.16[a] | 8.87±1.89[a] |
| P 5 | 25.85±4.17[a] | 8.06±0.01[a] | 8.60±2.22[a] |
| P 6 | 25.90±4.24[a] | 8.44±0.66[a] | 9.36±3.03[a] |
| P 7 | 25.90±4.24[a] | 8.59±1.29[a] | 9.94±4.19[a] |

Values with different letters on a single column proved significant difference through Kruskal-Wallis. Averages followed by ± standard deviation.

**Table 3.** Percent composition of the commercial feed 5 mm used for the experiment.

| Nutrient | Commercial guarantee (%)[1] | Tested value (%)[2] |
|---|---|---|
| Crude protein (min) | 32 | 32.87 |
| Ethereal extract (min) | 6 | 3.78 |
| Crude fiber (max) | 6.5 | 4.40 |
| Ashes (max) | 12 | 9.18 |

[1]Degrees of guarantee (%) according to the manufacturer. [2]Source: Laboratory of Food Analysis of the State University of Western Paraná.

**Table 4.** Mean values for the enzymatic activity of AST, ALT and FAL in the tilapia blood samples of the control treatment and product Homeopatila 100®.

| Enzymes (U/L) | AST | ALT | FAL |
|---|---|---|---|
| Control | 115.12 ± 75.03[a] | 18.51 ± 7.46[a] | 6.82 ± 3.44[a] |
| Homeopatila 100® | 104.41 ± 56.03[a] | 20.25 ± 8.97[a] | 7.55 ± 3.21[a] |

Each result is the average of 15 analyses (15 fish per treatment/3 per cage) with the respective estimates of standard deviation. Values with different letters on a single column proved significant difference through Student's t test ($p<0.05$).

pH and dissolved oxygen (Table 2) parameters between sampling locations ($p>0.05$).

### Percent composition of the commercial feed used for the experiment

Protein is the major visceral and structural component in the animal organism; therefore, its maintenance and production are very important (Furuya, 2010). Costa et al. (2009) assessed differences in the crude protein (CP) during the several growth phases of tilapia (*Chitralada*) cultivated in cages, with the most efficient diet for Nile tilapia being extruded feed with 32% crude protein. The feed used for the experiment meets the percentage considered ideal for the species (Table 3).

### Activity enzymatic: AST, ALT and FAL

The mean values for the enzymatic activity of AST, ALT and FAL of tilapia blood samples in the control treatment and the Homeopatila 100® presented no significant difference ($p>0.05$) according to Table 4.

### Levels of reduced glutathione (GSH)

Figure 3 illustrates the levels of reduced glutathione in the liver homogenate of tilapia in both the control and the homeopathic treatment. No difference ($p>0.05$) was indicated between the studied treatments for this parameter.

### Levels of carbonyl protein

No significant difference were observed ($p>0.05$) in the levels of protein oxidation in fish treated with Homeopatila 100® compared with the control group (Figure 3).

### Antioxidant enzymes

The catalase, superoxide dismutase, glutathione peroxi-

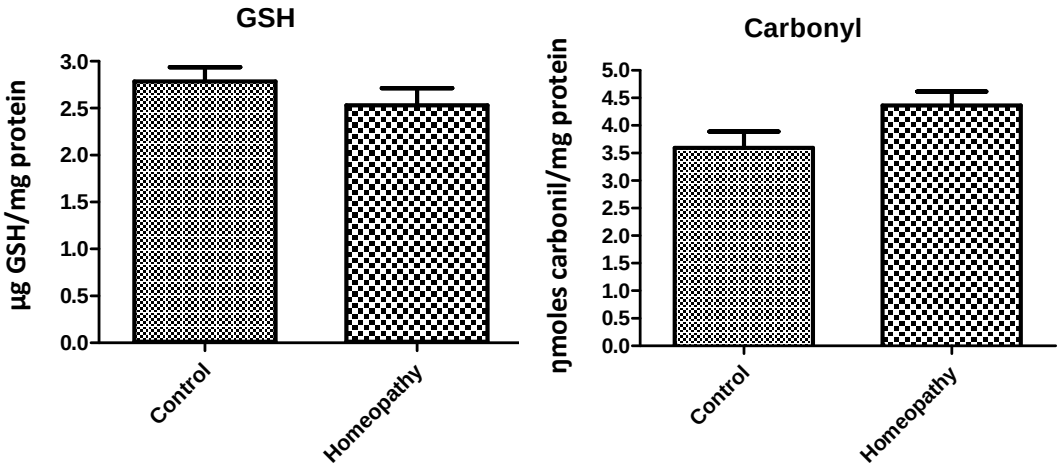


**Figure 3.** Levels of reduced Glutathione (GSH) and Carbonyl protein in tilapia livers.

**Table 5.** Enzymatic activity of catalase enzymes, superoxide dismutase, glutathione peroxidase and glutathione reductase in tilapia livers for the control group and homeopathic product.

| Enzymes | Control | Homeopatila 100® |
|---|---|---|
| Catalase (µmoles/min.mg protein) | 49.81±13.72[a] | 52.02±16.55[a] |
| Superoxide dismutase (USOD/mg protein) | 1.00±0.36[a] | 0.84±0.46[a] |
| Glutathione peroxidase (nmoles/min.mg protein) | 50.58±18.35[a] | 47.19±7.51[a] |
| Glutathione reductase (nmoles/min.mg protein) | 391.00±85.16[a] | 418.07±86.38[a] |

Each result is the average of 15 analyses (15 fish per treatment/ 3 per cage) with the respective estimates of standard deviation. Values with different letter on a single line proved significant difference through Student's t test ($p<0.05$).

peroxidase and glutathione reductase enzymes revealed no alterations in activity between the control and Homeopatila 100® as shown in Table 5.

### Assessment of muscle tissue

The mean values of the analyses of pH, color, texture and water retention capacity of the samples of both the control and Homeopatila 100® treatments are described as shown in Table 6. For pH, parameters of color a* and b* presented no differences between the studied treatments ($p>0.05$). The water retention capacity and texture were significantly higher ($p<0.05$) for the control, while luminosity was higher for the treatment with Homeopatila 100® ($p<0.05$).

## DISCUSSION

The purpose of this research was to assess whether using Homeopatila 100® for Nile tilapia in high density cages made improvements to the oxidative state and related parameters as well as the quality of the muscle tissue. This was in the light of results by Braccini et al. (2013) and Siena et al. (2010) who found a lower hepatosomatic index and histological analysis with higher amount of hepatocytes and the percentage for the indication of intracellular glycogen, presenting a more preserved liver morphologically for fish fed with using Homeopatila 100®.

Parameters related to water temperature, pH and dissolved oxygen (Table 2) are within normality according to Ribeiro (2001) for the cultivation of tropical fish such as Nile tilapia and presented similarity with the parameters found by Braccini et al. (2008) and Marengoni (2006).

As blood is a pathophysiological reflector of health of the entire body; consequently, it is an important factor regarding the diagnosis of the structural, functional conditions of fish. High levels of activity for the AST, ALT and FAL enzymes are excellent blood parameters for diagnosis of liver diseases (Motta, 2009). Firat et al. (2011) compared the effects of the exposing Nile tilapia to pesticides and metals by using enzymatic activities

**Table 6.** Assessment of muscle tissue quality in Nile tilapia.

| Parameter | Treatments | |
|---|---|---|
| | Control | Homeopatila 100® |
| Water retention[1] | 68.58±4.58[a] | 66.38±4.51[b] |
| pH[2] | 6.091±0.19[a] | 6.09±0.14[a] |
| **Color[3]** | | |
| Luminosity | 45.96±1.113[a] | 51.89±0.59[b] |
| a* (red/green) | 0.98±0.33[a] | 1.01±0.34[a] |
| b* (yellow/blue) | 1.35±0.50[a] | 0.955±0.33[a] |
| Texture/shearing force (N)[4] | 5.53±2.68[a] | 4.62±1.80[b] |

Values with different letter on a single line proved significant difference through Student's t test ($p<0.05$). Averages followed by ± standard deviation. [1]Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/in triplicate) with the respective estimates of standard deviation. [2] Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/ in triplicate) with the respective estimates of standard deviation. [3] Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/ in three different points of the fillet) with the respective estimates of standard deviation. [4] Each result is the average of 75 analyses (15 fish per treatment/ 3 per cage/ in five different points of the fillet) with the respective estimates of standard deviation.

(ALT, AST, and FAL) and found close values for these enzymes in their control group, corroborating the absence of alterations in these parameters for both treatments in the experiment (Table 4).

Homeopatila 100® indicated no influence for the parameters tested to assess oxidative state (Figure 3; Table 5) in Nile tilapia cultivated in high density (124 fish $m^3$). Sevgiler et al. (2004) found similar values for catalase (antioxidant enzyme) and superoxide dismutase, and higher values for glutathione peroxidase in the liver of tilapia of the control treatment by testing the effect of a pesticide in different concentrations. Likewise Braun et al. (2013) assessed the effect of combined manipulation with high stocking density in *Salminus brasiliensis* and verified that the enzymes related to oxidative stress suffered negative alteration through manipulation. Braun et al. (2010) also found that stocking density had a slight influence, indicating that stress is an important modulator of the antioxidant response. According to Cortez and Silva (2007), the imbalance of physiological processes generated by stress can be self-sustaining; a consequence of the process of cellular oxidative stress derived from the formation of free radicals (substances with high oxidant capacity). This is due to an irregular balance between the formation of these radicals and the capacity of response of the enzymatic arsenal of antioxidant defense of living organisms.

Several pre- and post-mortem factors can influence meat final quality such as pre-slaughter impacts such as the stress associated animal handling techniques (Stien et al., 2005; Koblitz, 2008). Color of flesh is one of the factors that can be influenced by animal stress. Myoglobin is a protein present in muscle tissue that forms the metamyoglobin when submitted to oxidation causing the brown color in meats. However, the darker the fish muscle, the less desirable it is to consumers (Thiansilakul et al., 2011). Fillets of the homeopathic treatment presented better luminosity in relation to the control treatment and therefore may be more saleable to the market.

The Ministry of Agriculture, Livestock and Food Supply (2001) through the regulation for sanitary and industry inspection of products of animal origin, established that the pH of fish must be under 6.5 inside the meat. The values found in our experiments for both treatments are in accordance with the recommendations. Soares and Gonçalves (2012) found similar values for pH (5.9 to 7.11) in Nile tilapia skinless fillets (*Oreochromis niloticus*).

Despite having presented higher water retention capacity in the fish muscle tissue, the control group presented lower shearing force for treatment with Homeopatila 100®. A higher value of shearing force corresponds to greater force required to break the sample; however, the tilapia fillets treated with homeopathy presented softer texture.

## Conclusion

Nile tilapia cultivated in high density receiving the homeopathic product Homeopatila 100® incorporated into their feed presented better-quality muscle tissues, especially concerning color ($L^*$ luminosity component) and texture compared with the control group-important factors for consumers' purchasing decisions. Homeopatila 100® did not indicate modifying effect on the oxidative state of Nile tilapia cultivated in high density.

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## Conflicts of interest

The authors state that there are no conflicts of interests.

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