Effect of dietary supplementation with astaxanthin on the hematological and biochemical response of Nile tilapias (*Oreochromis niloticus*)

Efeito da suplementação alimentar com astaxantina na resposta hematológica e bioquímica de tilápias do Nilo (*Oreochromis niloticus*)

Abstract

This study aimed to evaluate the effect of astaxanthin on the hematological, biochemical and somatic response of Nile tilapia (*Oreochromis niloticus*) orally administered in the feed for a period of 60 days. For the study, 105 tilapia (n=35) from the same spawn were used, constituting the following treatments: Control = animals (not treated with astaxanthin); T100 and T200 = fish treated with 100 and 200 mg of astaxanthin/kg of feed, respectively. There were no hematological alterations in red blood cells counts, hematocrit, hemoglobin, MCV and MCHC. In addition, astaxanthin-fed tilapia presented a better thrombocyte and leukocyte responses with a marked decrease in the number of thrombocytes, lymphocytes and neutrophils. Among treatments, there were no changes in serum levels of total protein, albumin, globulins, aspartate aminotransferase and alkaline phosphate. Treatment with astaxanthin resulted in a decrease in triglyceride and glucose levels, as well as increase in hepatosomatic indices. The results of hematological and biochemical analyzes of tilapias demonstrated the clinical safety of this carotenoid, not causing harmful effects to the health of fish. Therefore, the antioxidant activity of this compound in tilapias resulted in an improvement in the leukocyte profile and contributed to hypolipidemic effects at the dose of 200mg of astaxanthin/kg of feed.

Keywords: teleost fish, cichlids, astaxanthin, antioxidant, *Haematococcus pluvialis*, leukocytes.

Resumo

Objetivou-se avaliar o efeito da astaxantina na resposta hematológica, bioquímica e somática de tilápias do Nilo (*Oreochromis niloticus*) administrada via oral na ração por período de 60 dias. Para o estudo foram utilizadas 105 tilápias (n=35) oriundas da mesma desova, constituindo os seguintes tratamentos: Controle = animais (não tratados com *astaxantina*); T100= Animais tratados com 100mg de *astaxantina*/kg de ração; T200= Animais tratados com 200mg *astaxantina*/kg de ração. Não se observou alterações hematológicas na série vermelha (Red Blood cells, hematócrito, hemoglobina, VCM e CHCM), além disso, apresentou melhor resposta trombocitária e leucocitária com marcada diminuição no número de linfócitos e neutrófilos. Não ocorreu alterações nos níveis séricos de proteína total, albumina, globulinas, aspartate aminotransferase e fostatase alcalina. O tratamento com astaxantina resultou em diminuição nos níveis de triglicerídeos, glicose e aumento nos índices hepatossomáticos. Portanto, os resultados das análises hematológicas e bioquímicas das tilápias demonstraram a segurança clínica desse carotenóide, não causando efeitos nocivos à saúde dos peixes. No entanto, a atividade antioxidante desse composto em tilápias resultou em melhora do perfil leucocitário e contribuiu para efeitos hipolipemiantes na dose de 200mg de astaxantina / kg de ração.

Palavras-chave: peixe teleósteos, ciclídeos, astaxantina, antioxidante, *Haematococcus pluvialis*, leucócitos.

1. INTRODUCTION

Aquaculture is one of the fastest growing sectors in global agribusiness, with tilapia farming being the fourth species with the greatest economic interest (FAO, 2020). This growth has been associated with the demand for sanitary strategies in fish farms (Oliveira et al., 2021). The consumption of synthetic antioxidants can cause

adverse health effects. In this sense, the use of natural antioxidants is currently recommended as an alternative to synthetic antioxidants (Takyar et al., 2019). Antioxidant supplementation has become essential in aquaculture due to its ability to reduce the effects caused by stress (Belo et al., 2005; 2014) and repair oxidative damage in DNA, proteins or lipids. According to Smith et al. (2013), supplementation with astaxanthin in the fish diet improves meat color and neutralizes reactive oxygen species, being considered an effective antioxidant.

Astaxanthin is an antioxidant widely used in trout and salmon farms, as these animals are incapable of synthesizing carotenoids and pigments, requiring food supplementation (Johnson and An, 1991). Furthermore, this antioxidant modulates the immune system and offers protection against free radicals and infections, preventing cell damage, as well as having an anti-inflammatory effect (Park et al., 2010). Astaxanthin has an antioxidant activity about 500 times greater than α-tocopherols and 10 times greater than β-carotenes (Aracati et al., 2021), in addition to important nutraceutical properties such as increased immune responses and protection against various diseases.

Astaxanthin is the primary pigment responsible for the natural red color of wild salmon, lobster, crab and shrimp, and is used in salmonid fish feed to standardize the color of the meat and increase its commercial value (Dethlefsen et al., 2016). In humans and other mammals, this carotenoid has been shown to reduce oxidative stress and inflammation (Park et al., 2010).

Based on the socio-economic importance of intensive rearing of tilapia, associated with the benefits of using antioxidants in health management strategies of fish farms, this study aimed to evaluate the effect of dietary supplementation with

astaxanthin on the hematological, serum biochemical and somatic response of Nile tilapia (*Oreochromis niloticus*).

2. MATERIAL AND METHODS

2.1. Fish

For the astaxanthin study, 105 Nile tilapia (± 300g) from the same spawn (genetically improved strain, from Aquabel, Porto Ferreira/SP) were stored in 3 aquariums (n=35), with a capacity of 1000L of water each, supplied with chlorine-free running water from an artesian well, with a flow rate of 1 L/min. After being transported to the aquariums, the fish were acclimated for 15 days so that the plasmatic concentration of cortisol and osmolarity returned to basal levels. In the first three days of acclimatization, the animals were bathed in a NaCl solution at a concentration of 6.0 g/L (Carneiro & Urbinati, 2001). Water quality was determined at feeding times, with temperature and dissolved oxygen concentration measured by the YSI device, model 55, and pH and electrical conductivity by the YSI device, model 63. This research was approved by the Ethics Committee in Use of Animals (CEUA) under protocol No. 08360/19 of UNESP/FCAV.

2.2. Experimental Design

Fish were randomly distributed in 3 aquariums, constituting the following treatments: T0 (Control) = animals without astaxanthin treatment; T100= animals treated with 100mg of astaxanthin/kg of feed; T200= animals treated with 200mg of astaxanthin/kg of feed.

2.3. Experimental diet

Fish were fed a commercial diet (Nutripicis ® - Presence Company, containing 32% crude protein, 7,5 % ether extract, 12 % mineral matter and 4,5 % crude fiber). In treatments T100 and T200, 3% astaxanthin (Qingdao Vital Nutraceutical Ingredients, Bioscience Co., China) was added at a dose of 100 and 200mg/kg of feed, respectively. For diet preparation, the commercial feed was weighed and added with 2% of vegetable oil plus the respective amounts of astaxanthin, stored in dark plastic bags, kept at 7ºC until the moment of use. For standardization of diets, 2% of vegetable oil was added to the diet of control animals (T0). Feeding was carried out twice a day at 8:00 am and 5:00 pm.

2.3. Blood collection

After 60 days of feeding, tilapias were slaughtered by the hypothermia method with a mixture of water and ice in a 2:1 ratio, being carried out in a polystyrene cooler with a capacity of 120L. Fish were kept for approximately 3 minutes in the cooler for sensitization and blood collection. Subsequently, decerebration was performed in the brainstem for evisceration and morphological study of organs.

2.4. Hematological analysis

Blood samples were collected from the fish by caudal vessel puncture. The determination of the global count of red (HE) and white cells was carried out in a Neubauer chamber, using the solution of Natt and Herrick (1952) with a 1:100 diluent. The percentage of hematocrit (HT) was determined in a microcentrifuge and the amount of circulating hemoglobin (HG) with Drabkin's reagent for reading at a

wavelength of 540nm and the mean corpuscular volume (MCV) values were obtained by calculating MCV = (HT/HE)*100 and mean corpuscular hemoglobin concentration (MCHC) by calculating MCHC = (HG/HT)*100. Differential leukocyte counts were performed on blood extensions with a count of 200 cells, establishing the percentage of each cell type of interest, after previous staining of the extensions with May-Grünwald-Giensa-Wrigth (Farias et al., 2016).

2.5. Serum Biochemical

To carry out the serum biochemistry study, blood samples collected without anticoagulant were centrifuged at 10,000 rpm for five minutes to obtain serum, used for enzymatic and colorimetric determination of aspartate aminotrasferase (AST), alkaline phosphatase (ALP), total proteins, albumin, globulin, glucose, cholesterol and triglycerides in a semi-automatic biochemical analyzer (LabQuest Model – Bioplus) according to the methodology used by Belo et al. (2012).

2.6 Morphometric evaluation

Tilapia were slaughtered and eviscerated by the hypothermia method (item 2.3). Liver and spleen were collected for the following morphometric evaluation according Weibel et al. (1969), using the relationship between organ weight (OW) and body weight (BW). These were expressed as hepatosomatic index (HSI) and splenosomatic index (SSI), calculated using the formula: somatic index (SI) = OW × 100 / BW.

2.7 Statistical analysis

The experimental data were analyzed in a completely randomized design, using the SAS statistical program, PROC GLM procedure, version 9.3 (SAS, 2012). Multiple comparisons were measured by the Tukey Test at the 95% confidence level according to Snedecor and Cochran (1974).

3. RESULTS

3.1. Hematological analysis

Haematological evaluation of tilapia showed no significant difference (p> 0.05) among the treatments with astaxanthin and control for red blood cell counts, percentage of hematocrit, values of circulating hemoglobin, as well as for hematimetric values os MCV and MCHC (Table 1).

Table 1. Mean values[1] (±SE) and ANOVA[1] of hematological parameters in Nile tilápia treated with astaxanthin.

Treatments[2]	Total Erythrocytes (10^6/mm^3)	Hemoglobin (g/dL)	Hematocrit (%)	MCV [4] (fL)	MCHC [4] (g/dL)
Control	1.51 ± 0.06 A	6.95 ± 0.28 A	18.20 ± 1.95 A	108.20 ± 9.65 A	48.03 ± 6.11 A
T100	1.61 ± 0.06 A	7.48 ± 0.15 A	17.10 ± 0.54 A	108.90 ± 5.20 A	44.20 ± 1.16 A
T200	1.77 ± 0.08 A	7.26 ± 0.31 A	17.20 ± 0.44 A	99.05 ± 3.67 A	42.23 ± 1.28 A
Fvalue (Pr>F)[3]	2.03 NS	0.75 NS	0.16 NS	0.37 NS	0.34 NS
C.V.[3]	17.67	13.34	27.51	26.97	35.77

[1] Means (n=10) followed by the same letter in the column do not differ by Tukey test (P<0.05)

[2] Control - not treated with astaxanthin; T100 and T200 - Treated with 100mg and 200mg of Astaxanthin.

[3] CV - Coefficient of Variation (%); NS - Not significant; *significant for P<0.05; **Significant for P<0.01.

[4] MCV- Mean corpuscular volume; MCHC – Mean Corpuscular Hemoglobin Concentration.

The analysis of white blood cells showed a significant decrease (p <0.05) in the number of leukocytes in fish treated with 100 and 200 mg of astaxanthin when

compared to control animals (Table 2). Differential counts revealed that control animals presented a marked increase ($p<0.05$) in the number of lymphocytes and neutrophils, as well as significant increase ($p<0.05$) in the number of thrombocytes (Table 2).

Table 2. Mean values[1] (±SE) and ANOVA[1] of white blood cell counts in Nile tilapias treated with astaxanthin.

Treatments[2]	Total leukocytes µL	Thrombocytes µL	Monocytes µL	Lymphocytes µL	Neutrophils µL
Control	57026 ± 4029 ^{A}	37373 ± 4495 ^{A}	1108 ± 146 ^{A}	50224 ± 4308 ^{A}	5915 ± 738 ^{A}
T100	32928 ± 3672 ^{B}	10371 ± 1257 ^{B}	798 ± 106 ^{A}	28619 ± 3086 ^{B}	3589 ± 591 ^{AB}
T200	36067 ± 2404 ^{B}	11399 ± 473 ^{B}	908 ± 57 ^{A}	32940 ± 2127 ^{B}	2218 ± 360 ^{B}
Fvalue $^{(Pr>F)3}$	9.83**	21.71**	1.08 ^{NS}	8.31**	6.92 **
C.V.[3]	31.46	51.52	46.96	33.65	57.48

[1] Means (n=10) followed by the same letter in the column do not differ by Tukey test (P<0.05)

[2] Control - not treated with astaxanthin; T100 and T200 - Treated with 100mg and 200mg of Astaxanthin.

[3] CV - Coefficient of Variation (%); NS - Not significant; *significant for P<0.05; **Significant for P<0.01.

3.2. Biochemical analysis

The serum biochemical study revealed that tilapia treated with 200 mg of astaxanthin showed significant decrease ($p<0.05$) in serum glucose and triglyceride when compared to control fish (Table 3). There were no significant variations ($p>0.05$) in serum biochemical values of cholesterol, total proteins, albumin and globulins among the different treatments (Table 3). Treatments with 100 and 200 mg of astaxanthin did not result in significant disturbs ($p>0.05$) in the enzymatic activity of AST and ALP when compared to values observed in control animals (Table 3).

198 Table 3. Mean values[1] (±SE) and ANOVA[1] of serum biochemistry in Nile tilapias treated
199 with astaxanthin.

Parameters	Treatments[2]			Fvalue [(Pr>F)3]	C. V.[3] (%)
	Control	T100	T200		
Glucose (mg/dL)	65.66 ± 2.13 [A]	57.90 ± 1.43 [AB]	53.70 ± 2.67 [B]	4.48*	14.24
Cholesterol (mg/dL)	139.86 ± 5.98 [A]	161.38 ± 10.82 [A]	127.74 ± 10.38 [A]	2.33[NS]	24.68
Triglycerides (mg/dL)	227.88 ± 23.32 [A]	241.00 ± 15.37 [A]	120.91 ± 11.68 [B]	4.37*	37.20
Total Protein (g/dL)	3.88 ± 0.15 [A]	3.87 ± 0.09 [A]	3.63 ± 0.09 [A]	0.96[NS]	11.81
Albumin (g/dL)	1.50 ± 0.06 [A]	1.60 ± 0.07 [A]	1.40 ± 0.03 [A]	1.77[NS]	16.50
Globulin (g/dL)	2.38 ± 0.13 [A]	2.11 ± 0.09 [A]	2.23 ± 0.08 [A]	0.94[NS]	18.00
AST[4] (U/L)	70.99 ± 8.18 [A]	37.71 ± 4.70 [A]	52.96 ± 10.18 [A]	3.01[NS]	55.39
ALP[4] (U/L)	27.64 ± 1.17 [A]	29.85 ± 1.57 [A]	26.95 ± 0.98 [A]	0.94[NS]	16.83

200
201 [1] Means (n=10) followed by the same letter in the line do not differ by Tukey test (P<0.05)
202 [2] Control - not treated with astaxanthin; T100 and T200 - Treated with 100mg and 200mg of Astaxanthin.
203 [3] CV - Coefficient of Variation (%); NS - Not significant; *significant for P<0.05; **Significant for P<0.01.
204 [4] AST- Aspartate aminotrasferase; ALP- Alkaline phosfatase.
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206 **3.3 Somatic analysis**
207
208
209 Morphometric analyzes revealed an increase in the hepatosomatic index in
210 tilapia treated with 200mg of astaxanthin, and these findings were significant ($p<0.05$)
211 when compared to the indices observed in control fish (Figure 1). There were no
212 significant changes ($p>0.05$) in the splenosomatic index among treatments (Figure 1).
213

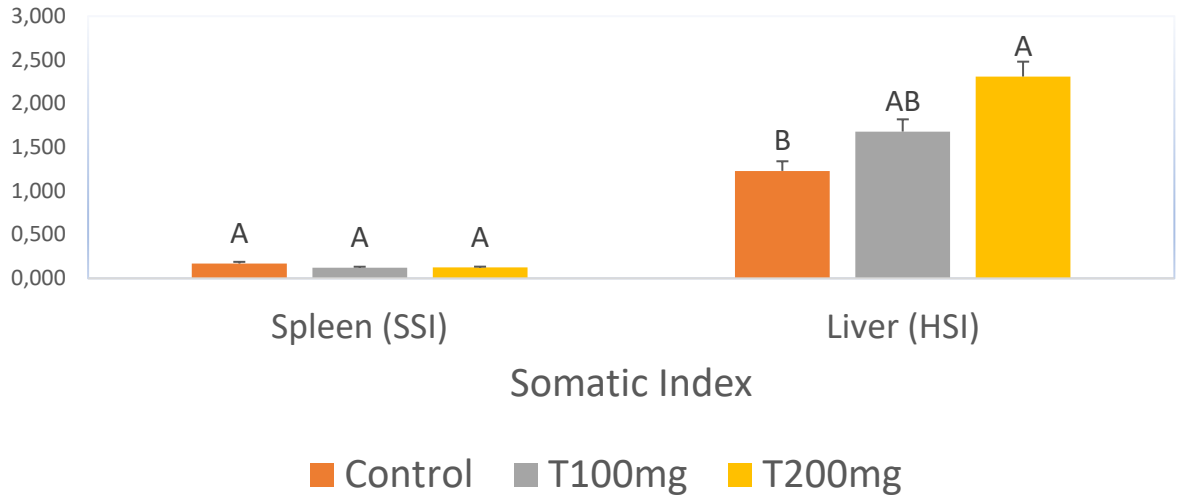

Figure 1. Mean values[1] (±SE) and ANOVA[1] of spleen (SSI) and liver (HSI) somatic index in Nile tilapias treated with astaxanthin. Means (n=10) followed by the same letter do not differ by Tukey test (P<0.05).

4. DISCUSSION

Dietary supplementation with 100 and 200 mg of astaxanthin/kg of feed for 60 days did not result in changes in hematological parameters of tilapia, demonstrating the clinical safety of this antioxidant, corroborating the findings of Lim et al. (2021) who observed significant improvements in hematological parameters (white and red blood cell counts, hemoglobin levels and percentage of hematocrit) in Asian sea bass, *Lates calcarifer*, supplemented with astaxanthin during *Vibrio alginolyticus* infection. According to Aracati et al. (2021), the hematological and biochemical parameters assist in the diagnosis and prognosis of morbid conditions. Pacus, Piaractus mesopotamicus, fed with diets deficient in vitamin E, another antioxidant, presented polycythemia and microcytosis (Belo et al., 2014).

The antioxidant effect of astaxanthin resulted in a decrease in the number of thrombocytes and leukocytes in tilapias, marked by a decrease in the number of

lymphocytes and neutrophils. These results suggest the hypothesis that fish supplemented with astaxanthin had a better leukocyte response, since modulation in circulating levels of white blood cells usually occurs during inflammatory defense responses (Moraes et al., 2018; Prado et al. 2018). Belo et al. (2014) observed a similar effect with improved leukocyte response in pacus supplemented with vitamin E during foreign body inflammatory reaction. In addition to participating in the blood clotting process, fish thrombocytes represent a link between innate and adaptive immunity, and these cells could be mobilized to contribute in organic defense mechanisms (Belo et al., 2013).

Dietary supplementation with 100 and 200 mg of astaxanthin/kg of feed did not result in changes in the serum enzymatic activity of AST, ALP, cholesterol, total protein, albumin, globulins, suggesting that astaxanthin has not caused damage in cytotoxicity and liver functionality. These findings corroborate the observations made by Lim et al. (2021) who reported an improvement in the serum biochemical profile of AST, ALT, glucose, cortisol, cholesterol and triglycerides of Asian sea bass, *Lates calcarifer*, supplemented with astaxanthin during *Vibrio alginolyticus* infection. Sheikhzadeh et al. (2012) studied the effects of astaxanthin supplementation on the serum biochemistry of rainbow trout (*Oncorchynchus mykiss*), and they did not observe changes in the values of serum alkaline phosphatase, AST, and total protein, suggesting that the doses used in the study were safe for this species of fish.

Pufferfish (*Takifugu obscurus*) supplemented with astaxanthin showed increased in sérum ALP activity, as well as decreased serum AST and ALT activity (Cheng et al., 2018). For these authors, dietary supplementation with astaxanthin resulted in improved resistance to oxidative stress in pufferfish. On the other hand,

257 elevated blood glucose is a common indicator of environmental stress in fish,
258 influenced under a variety of conditions (Sepici-Dinçel et al., 2009). Tilapias treated
259 with astaxanthin showed decreased blood glucose. Similar effects have been
260 described in rodents, where the use of astaxanthin has been shown to lower the blood
261 glucose level (Uchiyama et al., 2002; Naito et al., 2004; Hussein et al. 2007). During
262 stress responses, circulating glucose levels can vary significantly by activating
263 endocrine axes with the participation of catecholamines and corticosteroids
264 (Wandelaar-Bonga, 1997; Moonsen et al., 1999). The glucocorticoid activity of
265 endogenous cortisol proved to influence blood glucose levels and suppress the
266 defense responses of pacus, *Piaractus mesopotamicus*, acting on lymphocyte
267 populations that coordinate the release of inflammatory mediators such as cytokines
268 (Belo et al., 2012).

269 Changes in serum triglyceride levels may indicate liver dysfunction or lipid
270 metabolism disorder in response to physiological stressors (Cali et al., 2018). Serum
271 triglyceride analysis in tilapia revealed a significant decrease in tiapalia treated with
272 200mg of astaxanthin, showing the same trend as the results observed in the study of
273 blood glucose. These results together demonstrate an improvement in the energy
274 metabolism of fish raised under experimental conditions. Under stressful conditions,
275 elevated glucocorticoid levels result in increased energy demand that can often be
276 associated with increased serum levels of glucose and triglycerides (Wandelaar-
277 Bonga, 1997; Moonsen et al., 1999). These results corroborate the findings of other
278 studies that revealed the beneficial participation of astaxanthin in serum triglyceride
279 levels (Sheikhzadeh et al., 2012; Li et al. 2014; Lim et al., 2019). For these authors,
280 the antioxidant property of astaxanthin may be useful in alleviating hyperlipidemia

through triglyceride clearance mechanisms, which consequently mitigate stress in fish. The antihyperlipidemic potential of astaxanthin has been investigated in rat and mouse models with promising results (Ryu et al., 2012; Kumar et al., 2017). Astaxanthin also improves lipid metabolism in humans with hyperlipidemia (Yoshida et al., 2010; Choi et al., 2011).

The hepatosomatic index is used as a biomarker to identify possible liver disorders in fish (Narra et al., 2015). In the present study, fish treated with astaxanthin showed an increase in the hepatosomatic index, when compared to the control groups. Sadraddin et al. (2019) observed a non-significant increase in the hepatosomatic index of Cyprinus carpio supplemented with astaxanthin. Narra et al. (2015) studied the role of vitamin C in protecting the intoxication by the herbicide chlorpyrifos in *Clarias batrachus*, and they observed a reduction in the hepatosomatic index in the group exposed to the herbicide when compared to the group treated with Vitamin C. For thes authors, such fact indicate that the energy drain imposed by the stress to herbicide could be correlated with the loss of liver energy stores (Heath, 1995). A similar effect may have occurred in tilapias treated with astaxanthin, as the antioxidant effect of this compound was notorious on the circulating values of triglycerides. However histopathological studies of the liver tissue must be carried out in the future to understand these findings.

The results of hematological and biochemical analyzes of tilapias supplemented for 60 days with doses of 100 and 200mg of astaxanthin/kg of feed demonstrated the clinical safety of this carotenoid, not causing harmful effects to the health of fish. However, the antioxidant activity of this compound in tilapias resulted in an

improvement in the leukocyte profile and contributed to hypolipidemic effects at the dose of 200mg of astaxanthin/kg of feed.

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