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Production, egg quality, and intestinal morphometry of laying hens fed marine microalga

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ABSTRACT - The objective of the present study was to evaluate production and egg quality as well as the intestinal morphometry of laying hens fed diets supplemented with marine microalga *Dunaliella salina*. Six hundred laying hens were allocated based on a completely randomized design into five treatments (0, 0.25, 0.50, 0.75, and 1% inclusion of *D. salina* biomass) with 12 replicates of 10 hens per treatment. The experiment was divided into three periods of four weeks each, totaling 84 days. During this period, the productive performance of laying hens, the physical-chemical quality of the eggs, and the morphometric alterations of the small intestine and liver were determined. The inclusion levels of *D. salina* biomass had a linear effect on the performance (egg weight, egg mass, and feed conversion), qualitative parameters (yolk weight and yolk index), and physicochemical parameters of eggs (total carotenoids, TBARS, and yolk color). At the same time, villi lengths and the villus:crypt ratio of the duodenum and ileum segments and the metabolization of carotenoids in the liver were increased as an effect of *Dunaliella salina* dietary supplementation. Thus, the inclusion of marine microalgae *D. salina* biomass in experimental diets for laying hens improves the performance, the intestinal health, the physical-chemical quality of the eggs, and at the same time increases carotenoid content and improves egg oxidative stability.

Keywords: antioxidants, carotenoids, *Dunaliella salina*, liver, yolk pigmentation

1. Introduction

The search for foods that provide health benefits in addition to their basic nutritional functions, coupled with great supply and low acquisition cost, make the egg a potential functional food. Eggs contain a wide variety of essential nutrients and bioactive compounds that may contribute to human health (Miranda et al., 2015). With a mean calorie intake of 72 kcal, eggs are a good source of high biological-value protein, fatty acids, B vitamins, minerals, and choline and still provide less saturated fat per gram compared with other sources of animal protein (USDA, 2015).

To meet the demand of this new consumer market, the concept of enriched eggs was created. In this context, nutritional strategies are developed through the inclusion of raw materials, ingredients, or nutritional supplements in the poultry diet. Therefore, after the metabolism of these nutrients by laying hens, an egg production with higher nutritional value (Leeson and Caston, 2003).

The use of microalgae as an ingredient or feed additive has been increased in recent years and is one of the most frequently used food and feed supplement (Fraeye et al., 2012). The marine microalgae *Dunaliella salina* is a rich source of antioxidants, fatty acids, vitamins, minerals, and other bioactive compounds (Fimbres Olivarría et al., 2010) and at the same time has a high pigmentation due to its increased concentration of carotenoids (Ye et al., 2008; Fu et al., 2013). This property is also beneficial since egg yolk pigmentation is one of the most sought-after sensory attributes by consumers (Carvalho et al., 2006; Kljak et al., 2012).

Although *D. salina* has desirable levels of bioactive compounds that can be used in the nutrition of laying hens, the mechanisms of digestion, absorption, and metabolism of these nutrients by the birds and the patterns of deposition and egg enrichment may not be proportional to the inclusion level or period of dietary supplementation. Thus, it is necessary to develop research that can fill these gaps. Based on this principle, the objective of this study was to evaluate production and egg quality and the intestinal morphometry of laying hens fed this marine microalga.

2. Material and Methods

The experiment was approved by the Animal Use Ethics Committee (CEUA-23091.006877/2015-22) and conducted in a poultry house (5°11' S, 37°22' W, 16 m altitude). During the experimental phase, the average air temperature was 31.7 °C, with 63.8% humidity.

A total of 600 commercial laying hens of the Bankiva line were used during the period from 40 to 52 weeks of age, with an average weight of 1,800 g. The hens were confined in semi-close poultry house, allocated galvanized wire cages (25 × 40 × 30 cm), with nipple drinkers and trough type feeders, at a density of one laying hen per cage. During the experimental period, the chickens had free access to water and 120 g of diet/hens/day that was divided into two portions (offered at 08.00 and 16.00 h). No artificial light program was used. Therefore, the hens were subjected to 12 h of natural light.

The laying hens were distributed in a completely randomized design, with five treatments and 12 replicates of 10 hens per treatment. The experiment lasted 84 days and was divided into three periods of four weeks each. Periods 1, 2, and 3 were between the 40th and 44th weeks, 44th and 48th weeks, and 48th and 52nd weeks of age, respectively.

The treatments consisted of a control diet, without the addition of *D. salina* biomass, and the other diets further were supplemented with increasing inclusion levels of *D. salina* biomass, collected in solar saltworks on the Brazilian white coast, at the levels of 0.25, 0.50, 0.75, and 1%, by replacing the inert (sand). All diets were formulated according to the recommendations of Rostagno et al. (2011) for semi-heavy laying hens based on the medium production of egg mass (Table 1).

The performance parameters of laying hens were daily evaluated by means of feed intake (difference between the feed provided and the weight of leftovers, g/hens/day), laying rate (determined by dividing the number of eggs by the number of hens per treatment, %/hen/day), egg weight (g), egg mass production (calculated multiplying the average egg weight in the period by the laying percentage, g/hen/day), and feed conversion (calculated by dividing the feed intake by the egg weight, kg/kg).

For the evaluation of the production variables, whole eggs were collected daily in the last 72 h of each laying period, identified, and stored in a controlled temperature room (25 °C). The next day, they were weighed on a precision scale of 0.01 g to obtain the average weight (g).

The evaluation of egg quality was performed at the end of each week during the entire experimental period. For this, all eggs from each replicate were collected, and three eggs per replicate were selected to be randomly evaluated, avoiding broken or cracked eggs (Freitas et al., 2013). After being weighed, the eggs were carefully cracked to preserve the egg yolk integrity. The eggshells were washed and dried in an forced-air-circulation oven for 24 h at 55 °C.

The internal characteristics of the eggs were evaluated through the measurement of the diameter of the albumen and the yolk using a digital caliper. For height, the albumen and the yolk were broken on

a glass surface and measured by a micrometer of digital depth. The weight of the egg yolk and shell was measured through a precision scale of 0.01 g. The albumin weight was calculated by the difference between weights of egg, yolk, and shell.

The albumen height and egg weight data were used to calculate the value of the Haugh unit (HU), using the equation described by Pardi (1977): $HU = 100 \log (H + 7.57 - 1.7 W^{0.37})$, in which H is the height of the albumen (mm), and W is the weight of the egg in g. In turn, the yolk index was calculated by dividing the height of the yolk by its diameter (Lana et al., 2017).

The analysis of the egg yolk color was initially measured visually using the Roche Yolk Color Fan (DSM Nutrition Product, Basel, Switzerland). Subsequently, the yolk color was verified using a portable spectrophotometer (Konica Minolta Sensing Americas Inc., CM-700d, Ramsey, New Jersey, USA) programmed with the CIELab system considering the L*, a*, and b* coordinates, calibrated according to the manufacturer's manual. The color parameters of luminosity (L*), intensity of red (a*), and intensity of yellow (b*) were evaluated.

The total carotenoid content (mg/L) and pH were determined at the end of each laying period in three eggs per replicate that were randomly selected. A pool of yolk was extracted for carotenoid extraction

Table 1 - Ingredient composition and calculated nutrient content values of the experimental diets

Item	Experimental diet[1]				
	Control	D025	D050	D075	D1
Ingredient (kg)					
Soybean meal	27.00	27.00	27.00	27.00	27.00
Corn meal	55.00	55.00	55.00	55.00	55.00
Dunaliella salina biomass	0.00	0.25	0.50	0.75	1.00
Limestone	9.05	9.05	9.05	9.05	9.05
Dicalcium phosphate	1.80	1.80	1.80	1.80	1.80
Soybean meal	5.00	5.00	5.00	5.00	5.00
Sodium chloride	0.50	0.50	0.50	0.50	0.50
Vitamin and mineral premix[2]	0.10	0.10	0.10	0.10	0.10
Coccidiostat	0.03	0.03	0.03	0.03	0.03
L-lisine-HCl	0.37	0.37	0.37	0.37	0.37
DL-methionine	0.12	0.12	0.12	0.12	0.12
Inert (sand)	1.00	0.75	0.50	0.25	0.00
Nutrient (%)[3]					
Dry matter	89.64	89.64	89.65	89.65	89.65
Mineral matter	2.27	2.32	2.37	2.42	2.47
Ether extract	7.44	7.46	7.47	7.49	7.50
Neutral detergent fiber	10.28	10.29	10.29	10.30	10.30
Acid detergent fiber	4.04	4.04	4.04	4.04	4.04
Crude protein	16.87	17.01	17.16	17.30	17.45
Gross energy (kcal/kg)	2927	2937	2947	2957	2966
Digestible lisine	1.09	1.09	1.09	1.09	1.09
Digestible methionine	0.35	0.35	0.35	0.35	0.35
Calcium	3.92	4.08	4.24	4.40	4.56
Linoleic acid	3.92	3.94	3.96	3.98	4.00
Available phosphorus	0.32	0.35	0.37	0.39	0.41
Chlorine	0.34	0.34	0.34	0.34	0.34
Sodium	0.21	0.43	0.65	0.87	1.09
Potassium	0.65	0.98	1.30	1.63	1.95

[1] 0% (control), 0.25% (D025), 0.50% (D050), 0.75% (D075), and 1% (D1) of *D. salina* biomass inclusion.
[2] Guaranteed level per kilogram of product: vitamin A, 10,000,000 IU; vitamin D, 2,000,000 IU; vitamin E, 30,000 IU; vitamin K, 3 g; thiamine, 2 g; riboflavin, 2 g; pyridoxine, 6 g; cobalamin, 1.5 g; pantothenic acid, 12 g; folic acid, 1 g; biotin, 1 g; niacina, 5 g; iron, 100 g; selenium, 0.25 g; iodine, 2 g; manganese, 160 g; zinc, 100 g; vehicle qs.
[3] Calculated according to Rostagno et al. (2011).

according to Higby (1962) and homogenization of the albumen for pH measurement through a digital pH meter with an electrode and stainless penetration rod (in triplicate).

The lipid oxidation was evaluated by the determination of thiobarbituric acid reactive substances (TBARS) by the aqueous acid extraction method, according to Kang et al. (2001). At the end of each laying period, the eggs produced from the previous five days were collected, and five eggs were randomly selected per replicate. The egg yolks were separated from the albumen, placed in a beaker, homogenized, and taken for analysis. The content of reactive substances in the sample was expressed as milligrams of malonaldehyde per kilograms of yolk (Freitas et al., 2013).

At the end of the third period, after a 12-hour fast, one hen from each replicate was subjected to slaughter in a certified slaughterhouse following a commercial protocol, with electrical desensitization (55 volts for 10 s) and bleeding through a unilateral cut in the jugular vein and carotid artery. Subsequently, the segments of the small intestine and liver were collected.

For morphometric analysis of the intestine, 2-cm fragments of the medial portion of the three small intestine regions (duodenum, jejunum, and ileum) were collected, fixed, cut, and stained with hematoxylin and eosin, following standard histological processing (Junqueira and Carneiro, 2004). The sections were examined with an image analyzer, which allowed the evaluation of the effect of marine microalga *D. salina* on the intestinal epithelium and villus height. The villus height was measured from the basal villus, coinciding with the upper portion of the crypts, to its apex. The crypts were measured from their base to the crypt transition region:villi (Pelicano et al., 2003). The crypt depth:villus height ratio was determined by dividing the length of villi by the depth of the crypt.

The hepatic tissue was fixed, cut, and stained with hematoxylin and eosin (Junqueira and Carneiro, 2004). The liver slides were evaluated for possible saturation of hepatocytes by carotenoids, considering the changes present in the two samples of each hen (liver lobes).

The data obtained in the productive evaluation, quality, and physical-chemical parameters of the eggs were subjected to analysis of variance and, when there was a significant difference, to polynomial regression ($P<0.05$), according to the following model:

$$Y = B_0 + B_1X + B_2 X^2 + ... + B_kX^k + \varepsilon,$$

in which Y is the vector of the observations; X^1, X^2, ..., X^k the powers of X; B_1, B_2, ..., B_k are the parameters of the model; and ε is the vector of random errors (residuals).

To evaluate the saturation of hepatocytes by carotenoids, the Cramer-von Mises test (normality) and Chi-square test were used to test the independence of the variables through the frequencies and, later, Fisher's exact nonparametric test ($P<0.05$). The data were evaluated using the statistical software R (R Development Core Team, 2011).

3. Results

According to the nutritional composition of *D. salina* microalga (Table 2), it can be suggested that the microalga not only may be an effective additive, with high levels of total carotenoids, antioxidants, and pigments, but may partially replace the protein and energy sources of the laying hen diets.

In the three production periods, the inclusion of *D. salina* microalga biomass in the diets did not alter the performance of the hens as indicated by feed intake and egg production (Table 3).

There was a linear effect with the inclusion of *D. salina* biomass in the experimental diets (Table 3) for the parameters of weight (g) and egg mass (g) in laying periods 1, 2, and 3. There was no observed effect of the microalga inclusion in the diet on feed conversion in the first laying period (Table 3), while there was a linear effect in periods 2 and 3.

Regarding the qualitative parameters of the eggs (Table 4), no effects were observed with the inclusion of *D. salina* biomass in the diet on the shell weight, albumen, or HU in any of the evaluated laying periods.

The inclusion of the *D. salina* microalga in the experimental diets provided a linear effect on yolk weight and yolk index (Table 4) in laying periods 1, 2, and 3.

A linear effect was observed for the total carotenoid content of the egg yolk after the inclusion of *D. salina* biomass in the experimental diets (Table 5) for the laying periods 1, 2, and 3.

With the inclusion of *D. salina* biomass in the experimental diets, no effect was observed in the albumen pH in any of the evaluated periods (Table 5). The mean pH values observed for all treatments were within normal limits. The lipid stability in the yolk, as measured by TBARS values (Table 5), presented a linear effect with inclusion of biomass in laying periods 1, 2, and 3, indicating that the carotenoids present in the microalga were transferred to the eggs through the diet and were efficient in protecting the eggs against oxidative stress.

Table 2 - Nutritional composition of *D. salina* biomass

Nutrient (%)	*D. salina* biomass
Dry matter[1]	99.40
Crude protein[1]	58.60
Ether extract[1]	5.87
Neutral detergent fiber[1]	1.90
Acid detergent fiber[1]	0.38
Total carotenoids (mg/L)[2]	29.8
Beta-carotene[1]	10.0
Alpha-carotene[1]	3.5
Lutein, zeaxanthin, and cryptoxanthin[1]	2.0
Linoleic acid[1]	7.7
Gross energy (kcal/kg)[1]	3906

[1] Analysis performed according to AOAC (2011).
[2] Analysis performed according to Higby (1962).

Table 3 - Productive parameters of commercial laying hens fed increasing levels of *D. salina* biomass in experimental diets during three laying periods (28 days each)

Productive parameter	*D. salina* biomass inclusion in diets (%)					Statistical analysis		
	0	0.25	0.50	0.75	1.00	CV (%)	SE	P
First period (40 to 44 weeks)								
Feed intake (g/hen/day)	107.73	108.66	108.06	108.33	108.73	7.88	3.11	0.8880
Laying rate (%)	85.95	87.61	87.14	87.61	88.81	9.65	3.93	0.6791
Egg weight (g)[1]	53.27	56.60	57.80	59.80	61.60	8.99	2.39	0.0001
Egg mass (g)[1]	46.16	49.05	51.33	53.96	55.44	7.34	2.09	0.0001
Feed conversion ratio (kg/kg)	2.03	1.93	1.87	1.81	1.76	9.56	0.09	0.6500
Second period (44 to 48 weeks)								
Feed intake (g/hen/day)	108.60	108.80	108.26	109.07	108.93	7.85	2.55	0.6555
Laying rate (%)	86.19	87.25	87.34	87.48	87.86	9.90	4.23	0.4511
Egg weight (g)[1]	56.87	57.93	60.27	61.87	64.00	13.99	2.77	0.0001
Egg mass (g)[1]	47.16	50.76	52.52	54.21	56.84	7.03	2.46	0.0001
Feed conversion ratio (kg/kg)[1]	1.98	1.88	1.79	1.77	1.71	9.08	0.08	0.0001
Third period (48 to 52 weeks)								
Feed intake (g/hen/day)	108.87	109.47	109.07	108.60	108.80	7.15	2.03	0.5472
Laying rate (%)	86.90	86.67	86.79	83.33	88.81	9.76	3.76	0.0791
Egg weight (g)[1]	57.13	58.20	61.33	62.66	65.80	8.16	1.93	0.0001
Egg mass (g)[1]	49.24	50.16	52.43	53.57	57.81	7.29	1.80	0.0001
Feed conversion ratio (kg/kg)[1]	1.91	1.88	1.78	1.73	1.66	7.87	0.06	0.0001

CV - coefficient of variation; SE - standard error; P - probability.
[1] Linear effect (P<0.05).

Table 4 - Qualitative egg parameters of commercial laying hens fed increasing levels of *D. salina* biomass in experimental diets during three laying periods (28 days each)

Qualitative parameter	*D. salina* biomass inclusion in diets (%)					Statistical analysis		
	0	0.25	0.50	0.75	1.00	CV (%)	SE	P
First period (40 to 44 weeks)								
Shell weight (g)	7.50	6.83	8.17	7.50	7.0	5.80	0.95	0.7872
Yolk weight (g)[1]	15.66	16.06	17.00	18.53	19.93	9.45	0.99	0.0001
Albumen weight (g)	30.33	35.50	32.33	33.17	35.00	6.15	2.64	0.3865
Yolk index[1]	0.39	0.42	0.45	0.48	0.51	4.84	0.01	0.0001
Haugh unit	89.99	90.86	91.69	90.32	89.75	7.65	3.02	0.6773
Second period (44 to 48 weeks)								
Shell weight (g)	6.17	6.83	6.67	7.00	7.50	4.67	0.99	0.8480
Yolk weight (g)[1]	15.40	16.47	17.13	18.73	20.00	4.30	0.92	0.0001
Albumen weight (g)	32.67	34.50	36.00	37.33	34.33	7.10	2.66	0.0833
Yolk index[1]	0.39	0.42	0.45	0.48	0.51	4.80	0.01	0.0001
Haugh unit	90.89	91.09	90.76	90.09	90.69	9.60	2.89	0.5530
Third period (48 to 52 weeks)								
Shell weight (g)	8.50	7.17	7.17	8.16	7.33	5.65	1.09	0.3538
Yolk weight (g)	15.60	17.00	17.73	18.93	20.33	8.50	0.81	0.0001
Albumen weight (g)	30.83	33.17	36.67	35.00	33.50	6.90	2.75	0.0534
Yolk index[1]	0.39	0.42	0.45	0.49	0.51	4.20	0.01	0.0001
Haugh unit	91.93	91.28	90.99	90.13	90.19	8.89	2.89	0.0536

CV - coefficient of variation; SE - standard error; P - probability.
[1] Linear effect (P<0.05).

Table 5 - Physicochemical parameters of eggs of laying hens fed increasing levels of *D. salina* biomass in experimental diets during three laying periods (28 days each)

Physicochemical parameter	*D. salina* biomass inclusion in diets (%)					Statistical analysis		
	0	0.25	0.50	0.75	1.00	CV (%)	SE	P
First period (40 to 44 weeks)								
Total carotenoids[1]	190.1	246.6	323.1	390.5	411.3	2.53	4.02	0.0001
Ph	7.55	7.70	7.62	7.67	7.71	10.81	0.16	0.1642
TBARS[1]	0.98	0.91	0.85	0.79	0.64	3.78	0.02	0.0001
Yolk color fan[1]	5.33	7.17	14.33	15.0	15.0	3.40	1.95	0.0001
L*	54.24	56.12	52.28	50.64	49.10	5.20	4.30	0.1667
a*[1]	3.69	19.67	27.71	29.99	31.16	4.85	4.99	0.0001
b*	34.15	40.99	35.30	35.81	33.16	4.60	4.55	0.2338
Second period (44 to 48 weeks)								
Total carotenoids[1]	189.1	250.3	339.3	399.5	421.9	7.26	6.50	0.0001
Ph	7.63	7.68	7.68	7.70	7.63	1.98	0.17	0.9384
TBARS[1]	0.97	0.91	0.85	0.79	0.65	4.13	0.02	0.0001
Yolk color fan[1]	5.33	7.17	14.33	15.0	15.0	3.80	1.95	0.0001
L*	53.21	46.30	49.48	49.63	45.19	4.90	7.12	0.1779
a*[1]	4.41	21.81	25.89	28.10	32.37	3.85	4.39	0.0001
b*	32.45	31.68	33.10	37.13	26.64	4.35	5.49	0.3906
Third period (48 to 52 weeks)								
Total carotenoids[1]	162.6	261.9	344.5	403.1	430.1	3.56	6.25	0.0001
pH	7.57	7.68	7.78	7.73	7.77	9.33	0.14	0.4198
TBARS[1]	0.99	0.90	0.85	0.78	0.64	3.18	0.02	0.0001
Yolk color fan[1]	6.33	13.5	15.0	15.0	15.0	3.70	2.19	0.0001
L*	62.71	52.68	49.14	51.50	49.79	4.55	4.06	0.1774
a*[1]	3.47	24.34	30.94	32.07	33.95	3.90	6.17	0.0001
b*	36.97	35.46	35.67	36.64	33.71	4.28	2.66	0.1313

TBARS - thiobarbituric acid reactive substances; L*, a*, and b* - luminosity, intensity of red, and intensity of yellow, respectively; CV - coefficient of variation; SE - standard error; P - probability.
[1] Linear effect (P<0.05).

Regarding yolk color (Table 5), a linear effect was observed in the response to the inclusion of the microalga in the experimental diets in laying periods 1, 2, and 3. It was also observed that in periods 1 (40 to 44 weeks) and 2 (44 to 48 weeks), the yolk color reached the maximum value of the colorimetric fan in eggs of the 0.75% inclusion group. In the third period (48 to 52 weeks), the value was also reached in the 0.50% inclusion group.

On the contrary, the color determined by the CIELab system responded linearly to the inclusion of biomass in the diets for red intensity (a*) in the 1st, 2nd, and 3rd laying periods. The predominance of the high values of a* observed for treatments with the inclusion of *D. salina* (0.25, 0.50, 0.75, and 1%) gave the yolk a color ranging from orange (0.25%) to red-orange (1%).

The inclusion of *D. salina* biomass in experimental diets for laying hens provided a linear increase in intestinal villus height and the crypt depth:villus height ratio (Table 6) in the duodenum segments and ileum. The antioxidants present in the *D. salina* microalga stimulated cell turnover in this segment of the intestinal mucosa, with higher villus length observed in the 1% inclusion group.

No effects of the inclusion of *D. salina* biomass in diets on crypt depth were observed in any of the small intestinal tracts (Table 6).

Through the histological observation made in the liver of laying hens, a relationship of saturation of hepatocytes with carotenoids was observed with the inclusion of *D. salina* biomass in diets (Table 7).

Table 6 - Intestinal morphometry of commercial laying hens fed increasing levels of *D. salina* biomass in experimental diets

Intestinal morphometry	*D. salina* biomass inclusion in diets (%)					Statistical analysis		
	0	0.25	0.50	0.75	1.00	CV (%)	SE	P
				Duodenum				
Villus height (µm)[1]	801.2	993.1	1108.7	1236.8	1353.7	12.99	142.8	0.0001
Crypt depth (µm)	228.7	261.6	231.6	206.8	202.3	16.61	39.35	0.0676
CD:VH[1]	3.52	3.85	4.92	6.47	6.81	14.72	1.27	0.0001
				Jejunum				
Villus height (µm)	707.5	735.4	789.2	879.2	891.9	12.93	178.5	0.9433
Crypt depth (µm)	258.9	249.1	192.3	198.4	184.2	17.51	59.58	0.5229
CD:VH	2.74	3.02	4.25	5.22	5.79	11.35	1.79	0.8950
				Ileum				
Villus height (µm)[1]	415.9	587.8	665.2	842.3	938.0	7.80	56.4	0.0001
Crypt depth (µm)	100.7	105.1	119.6	145.3	111.2	15.96	30.22	0.0684
CD:VH[1]	4.01	5.72	6.85	7.05	9.05	11.93	0.71	0.0001

CD:VH - crypt depth:villus height ratio; CV - coefficient of variation; SE - standard error; P - probability.
[1] Linear effect (P<0.05).

Table 7 - Saturation of liver hepatocytes of commercial laying hens fed increasing levels of *D. salina* biomass in experimental diets

D. salina biomass inclusion in diets (%)	Saturation of hepatocytes by carotenoids[1,2]				P
	Yes		No		
	n	%	n	%	
0 (control)	0	0	24	100	
0.25	9	37.50	15	62.50	
0.50	15	62.50	9	37.50	0.0255
0.75	19	79.16	5	20.83	
1.0	24	100	0	0	

n - number of livers observed; P - probability.
[1] Data subjected to the Chi-square test of Pearson's independence and subsequent Fisher's exact test.
[2] Saturation of hepatocytes by carotenoids has an increasing linear relationship with inclusion levels of *D. salina* biomass in the laying diet according to Fisher's exact test (P<0.05).

4. Discussion

Voluntary feed intake in poultry is directly related to palatability (Leeson and Summers, 2001; Freitas et al., 2013), fibrous fraction, and energy content of the diet (Fernandes et al., 2015). As indicated, there was no significant difference for the feed intake, and it could be concluded that the addition of increasing levels of *D. salina* biomass did not significantly influence the palatability, gross energy, or fibrous fraction values of the experimental diets.

Although *D. salina* is a microalga of high protein value, and its inclusion in the diets enhanced the crude protein content of the diets, this increase alone was not enough to improve egg production. A possible explanation is the fact that the diets were isonutritive, and a similar feed intake of the experimental diets was observed. Similar results were observed by Pavan et al. (2005), when they evaluated the effects of different levels of crude protein and sulfur amino acids on the performance of laying hens and did not observe changes in the laying rate.

This increase in egg weight and mass could be attributed to the higher content of carotenoids and lipid compounds available to laying hens through the inclusion of *D. salina* biomass in rations, which were absorbed into the intestine and then transferred to the liver and tissues through the portomicrons and the egg yolk by the lipoproteins modified for deposition in the yolk (Bjorneboe et al., 1990; Rocha, 2011). These compounds present in high quantities increased the weight of the yolk and, consequently, the weight of the egg, which is also a key factor to determine the egg mass.

As feed conversion is a parameter that depends on feed intake and egg weight, the fact that the biomass inclusion did not affect feed intake resulted in lack of significant effects for the conversion rate in the first laying period. The increase in egg weight observed for all treatments in periods 2 and 3, considered a natural consequence of the advancing age of hens (Garcia et al., 2010), induced an improvement in feed conversion according to the increasing levels of *D. salina* in diets.

The absence of a significant influence of natural sources of antioxidants and bioactive compounds on qualitative parameters of eggs, such as bark and albumen weight, is common (Özek et al., 2011; Freitas et al., 2013), mainly due to the low levels of their inclusion in the diets of laying hens. It is known that the HU is a measure that correlates albumen height and egg weight, and the higher the value obtained, the higher the quality of the egg. Values greater than 70 are characteristic of fresh eggs or indicate low protein and lipid oxidation rates (Figueiredo et al., 2011).

The use of antioxidants in poultry diets delay the natural process of oxidation of egg components; however, they do not have a direct effect on the albumen and thus on the HU, as could be observed in this study. All evaluated eggs presented HU above 70, which is associated with fresh and excellent-quality eggs (USDA, 2000).

It was also observed that the subsequent laying periods presented higher total carotenoid content in the treatments with microalga inclusion, indicating that the concentration of these compounds in the yolk occurred progressively. Carotenoids are compounds of great dietary importance and cannot be synthesized by animals and, therefore, must be obtained from the diet from both natural and synthetic sources (Garcia et al., 2002; Breithaupt, 2007).

These compounds act not only as precursors of vitamin A but also as molecules that participate in the mechanisms of cellular protection. Carotenoids influence the color of foods, which is one of the first attributes perceived by the consumer, directly influencing his/her choice (Rao and Rao, 2007). Considering the above, the inclusion of *D. salina*, a natural source of carotenoids, had a positive effect on the concentration of this compound in the yolk.

According to Barancelli et al. (2012), the eggs begin to show an increase in pH of the albumen 72 h after laying, from 7.8 to 9.6, due to the loss of carbonic acid, which is one of the components of the albumen buffer system. Thus, as the eggs were evaluated 24 h after laying, the dissociation of this acid into water and carbon dioxide, with the consequent release of the gas into the medium through the egg pores, was not significant enough to alter the pH, or for some antioxidant compound present in the microalga biomass to influence the obtained values.

The results observed for L*, a*, and b* were in accordance with the findings of Carvalho et al. (2006), who evaluated the influence of dietary supplementation of marine carotenoids on egg yolk pigmentation of laying hens. The authors observed that the addition of algae to the diet, from 0.50 to 1.75%, provided progressive pigmentation of the yolk evaluated by β-carotene equivalents and visual score. In addition, the result observed ratified the effect for the total carotenoids present in the yolk, since the higher deposition of these compounds resulted in a higher pigmentation (Breithaupt, 2007).

On the other hand, the absence of an effect on L* and b*, as well as the strong pigmentation in the shade of red (a*), may be an indication that the carotenoids incorporated to the yolk were predominantly carotene precursors of vitamin A, such as β-carotene (Tallarico et al., 2002), constituent in 10% (dry basis) of the microalgae used in this study.

The development of the intestinal mucosa consists of increasing the height and density of the intestinal villi, which corresponds to the increase in the number of their epithelial cells (enterocytes, goblet cells, and enteroendocrine cells) and, therefore, to the increase in digestive capacity and intestinal absorption (Zavarize et al., 2011). Some compounds act as trophic agents in the intestinal mucosa; that is, they stimulate the mitotic process in the villus:crypt region and, as a consequence, increase the number of cells and the size of intestinal villi (Macari et al., 2002). Thus, due to the fact the *D. salina* biomass is a source of carotenoids (Fimbres Olivarría et al., 2010) and has a lipidic nature, the linear effect of diet on villus length observed was justified.

It is emphasized that along the small intestine, the villus density and length, as well as the turnover rate, were not the same. The duodenum, in addition to having a higher villus height, also has the highest cell turnover rate, since it is the first segment of the intestine to receive physical, chemical, and hormonal stimuli with the presence of food in the intestinal lumen (Maiorka et al., 2002). In contrast, in the jejunum portion, the cell turnover rate tended to be naturally lower, which justifies the absence of a dietary effect on the villi in this segment. The ileum is the final portion of the small intestine, and the resorption of bile salts responsible for the solubilization of the lipid molecules occurs in this segment (Macari et al., 2002).

The depth of the crypt is an indication of the compensatory capacity or hyperplasia of the epithelial cells due to a higher level of aggression to the morphological structure of the intestinal mucosa caused by the diets (Arruda et al., 2008). As no effect was observed in this parameter, abrasive components in the diets provided to the laying hens in this study were not possibly present. In turn, the increase in the villi:crypt ratio indicated an adequate cell turnover rate and a higher absorptive capacity, demonstrating the positive effect of microalga inclusion in the diets.

Since hepatocytes actively participate in the metabolism of carotenoids and transport them to the yolk, the saturation of these cells corroborates with the linear effect for the carotenoid content in the yolk observed in this study. The lipid fraction of the diet, including carotenoids, is absorbed by enterocytes in the small intestine, where they migrate to the endoplasmic reticulum (from enterocytes) for complex lipid biosynthesis. The carotenoids are quite hydrophobic, requiring involvement in portomicrons that are released by enterocytes in the portal vein and go to the liver. Once in the liver, hepatocytes secrete modified lipoproteins for deposition into the yolk that will transport these carotenoids to the egg yolk (Champe et al., 2006).

Although saturation of hepatocytes is visible with increasing levels of *D. salina* biomass in experimental diets, this saturation was not associated with hepatic steatosis, demonstrating the viability of this source of carotenoids as a pigment without negatively influencing the health status of laying hens.

5. Conclusions

The inclusion of biomass of the *D. salina* marine microalga in experimental diets for laying hens improves performance, intestinal health of the hens, and physicochemical quality of the eggs, as well as induces a higher carotenoid content, egg yolk color, and protection from lipid oxidation. The inclusion of *D. salina* biomass can be safely used for a period of 84 days in laying hens diets without impairing the hepatic function of the poultry.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Data curation: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Formal analysis: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Funding acquisition: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Investigation: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Methodology: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Project administration: A.A. Gonçalves and A.M.V. Arruda. Resources: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Supervision: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Validation: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Visualization: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Writing-original draft: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda. Writing-review & editing: R.T.V. Fernandes, A.A. Gonçalves and A.M.V. Arruda.

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