Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2023 / v.25 / n.4 / 001-012

http://dx.doi.org/10.1590/1806-9061-2022-1753

Original Article

Effect of Inclusion of Arthrospira maxima Microalgae in Laying Hen Diets on Production Parameters and Egg Characteristics

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Keywords

Spirulina; poultry; feedstuff; Egg Quality; fatty acids.

Submitted: 15/December/2022
Approved: 11/July/2023

ABSTRACT

The animal feed industry is continuously researching new feed additives to substitute other materials, reduce costs, or add value to the final product. The microalgae *Arthrospira maxima,* cultivated using wastewater as a nutritional source, was evaluated as a feed additive by including 2, 4, and 6% in isocaloric and isoprotein diets for laying hens. Five replicates per treatment with 5 hens per cage were used during an experimental period of 28 days. Productive behavior and egg characteristics (quality, fatty acid profile, cholesterol level) were evaluated. The inclusion of microalgae up to 4% in diets for 52-week-old laying hens did not affect productive performance (egg production, egg weight, egg mass, FCR, shell thickness, and Haugh units). No effects were observed on the cholesterol level or the concentration of fatty acids in the eggs, but more information is needed to determine if the microalgae drying or storage process can generate variations of these results. The inclusion of 6% microalgae produced an acceptable egg yolk color for the local market. The results indicate that this material can be used as a protein source up to an inclusion of 4% in the diet, and hens may need an adaptation period to maintain production at the 6% inclusion level.

INTRODUCTION

The demand for soy and maize for animal feed production has accelerated in the last 5 years to support the increasing demand for protein sources for animal production. In 2021, 195.5 thousand metric tons of soy were produced in South America, with approximately 75% destined for animal feed (Cargill, 2021). The Chamber of Balanced Food Industrialists (CIAB by its Spanish acronym) showed in their annual report (2021) a production of 142,533 metric tons of poultry feed in 2020 to sustain the production of 3.55 million laying hens, mainly made from imported sources such as maize and soy. Therefore, there is a constant need to evaluate alternative feed sources to reduce dependence on these resources and add value to local production. A new trend has emerged for animal protein production using other feed and additives capable of being as efficient as antibiotics, adding pigments or other biomolecules (Mariey *et al.*, 2012), such as microalgae. In literature, the inclusion of the marine microalgae *Dunaliella salina* in the diets of laying hens improved performance, intestinal health, physicochemical quality of eggs, increased carotenoid content, and improved egg oxidative stability (Fernandes *et al.*, 2020). In Japanese quails, birds fed with the microalgae *Arthrospira platensis* increased yolk index, color intensity, antioxidant capacity, and mono-unsaturated fatty acid levels of the yolks; decreased the levels of saturated and polyunsaturated fatty acids in egg yolks, and levels of lipid peroxidation were also lower (Boiago *et al.*, 2019).

Among the alternative microalgae species, *Arthrospira maxima* and *A. platensis* (commercially known as spirulina) can be produced autotrophically, heterotrophically in the absence of light with an organic carbon source (i.e., glycerol) and, mixotrophically (carbon and nutrients source derived from excreta and wastewater) (Ortiz-Moreno *et al.*, 2012). The main advantage of mixotrophic microalgae cultivation is the reduction in production costs by up to 80% due to a decrease in inorganic nutrient supply in culture media. This suggests an advantage in industrializing cultivation as a component of circular economies in livestock production (i.e., pig or chicken farms) and obtaining nutrient-rich biomass as a food resource (Pereira *et al.*, 2019). *A. maxima* has been perceived as a feed supplement, as it has demonstrated beneficial functional properties in animal health, and its nutritional characteristics are complementary to soybean meal (Alvarenga *et al.*, 2011).

Studies have reported on the palatability, toxicity, digestion, antioxidant action, pigmenting capacity, anticancer properties, immune stimulation, and anti-inflammatory capacity of these microalgae in rabbits, rats, chickens, and pigs (Belay *et al.*, 1996; Grinstead *et al.*, 2000; Rodrıguez-Hernández *et al.*, 2001; Derner *et al.*, 2006; Colla *et al.*, 2007; Ambrosi *et al.*, 2008; Peiretti & Meineri, 2011; Oliveira *et al.*, 2013; Moreira *et al.*, 2015). All these properties are associated with the presence of biomolecules in *Arthrospira maxima,* such as phycocyanin, phenolic compounds, polyunsaturated fatty acids, carotenoids, and the antiviral calcium spirulan (Parra *et al.*, 2017). Likewise, there are reports of this microalgae's ability to color chicken egg yolks at the same level as commercial colorants without affecting the laying hens' productive performance (Anderson *et al.*, 1991; Zahroojian *et al.*, 2011; 2013)

In most of these studies, microalgae biomass was produced under autotrophic and heterotrophic conditions; however, little information is available on the effect of algae produced by mixotrophs as a feed additive on animal performance and diet cost. Recent studies have shown that mixotrophic cultivation of *Arthrospira* sp. in wastewater induces carbohydrate production and modification of its lipid profile (Hena *et al.*, 2018; Pereira *et al.*, 2019). This study aimed to evaluate the inclusion of *Arthrospira maxima* obtained under mixotrophic conditions in diets for laying hens and its effect on productive performance and egg characteristics.

MATERIALS AND METHODS

The study was carried out at Los Pollitos poultry farm, located in the Zaragoza district of Alajuela province, Costa Rica, with minimum and maximum ambient temperatures of 17°C and 29°C (Costa Rican Meteorological Institute, 2019). A total of 100 ISA Brown laying hens, aged 52 weeks, were used. The laying hens were housed at room temperature, under a 16-hour lighting program, in battery cages with three levels (dimensions of the cage 30 x 30 x 80 cm), with 5 laying hens per cage (experimental unit), with 5 random replicates per treatment, for a total of 20 experimental units. The trial was approved by the Animal Use Ethics Committee of the University of Costa Rica (CICUA). The experimental period was 28 days and normal farm management was continued throughout the trial. The laying hens were fed daily, with a feed offer of 700 grams per cage at 6:00 am, and water was offered ad libitum through 2 nipples per cage. Feed residues (if any) were measured once a week for feed conversion ratio calculation, which considered the weekly mass of feed consumption per cage divided by the mass of eggs produced.

Microalgae biomass and diets

The biomass of the microalgae *Arthrospira maxima* was produced at the Biotechnology Research Center of the Costa Rica Institute of Technology (TEC, by its Spanish acronym). The culture medium was formulated from a wastewater dilution obtained from a pig farm, previously conditioned by anaerobic digestion for 27 days, and treated with sodium hypochlorite, which was later neutralized with sodium metabisulfite before inoculating the microalgae. The culture medium was supplemented with 10 g/L of sodium bicarbonate as a source of carbon and for pH regulation range (9-10). The initial inoculum was kept at 0.1 g/L of culture (17,000.00 cells/mL) and the chemical conditions of the culture medium were measured with QUANTOFIX® Relax plus kits (Machery-Nagel) and maintained over time at <200 mg/L SO42-, <4100 mg/L PO43-, <80 mg/L NH4+, <550 mg/L NO3-, and <400 mg/L K+. The microalgae culture was carried out in a continuous raceway system with a water level of 0.4 m deep and a volumetric capacity of 30,000 L. The culture was monitored for 35 days before downstream; cell growth was followed under an optical microscope (Leica DM 750) with a Neubauer cell counting chamber. Dry weight was measured using a halogen thermo-balance (Radwarg PNR 50). The ideal biomass density for harvesting was presented on day 35, reaching 0.71

g/L. A high culture yield of 0.98 g/L was obtained in the continuous phase while 30% of the culture was harvested weekly by centrifugation at 4200 rpm (Gea Westfallia SSD 606007 separator) and dried by spraying (Galaxie ECO Dryer® 1512) to obtain a fine green powder.

In vitro dry matter digestibility (Van Soest & Robertson 1979), microorganisms, and heavy metals in the biomass were analyzed as a qualitative assessment prior to designing feeding experiments.

Using the nutritional information of the algae (Table 1), four experimental diets were formulated using the Brill® software to meet the nutritional requirements of ISA Brown laying hens based on the Nutritional Guide for ISA Brown (Hendrix Genetics 2020b). The following microalgae nutrients were determined using AOAC methods (AOAC 2005) moisture 930.15, crude protein 990.0, crude fiber 962.09, ether extract 920.39, salt 969.10, ash 942.05, calcium 975.03, 968.08, phosphorus 965.17, 986.24, and sodium 985.35. Nitrogen-free extract was determined according to Morrison (1950) and total amino acids were determined according to Bartolomeo Maisano (2006)

Table 1 – Nutritional composition of *Arthrospira maxima* biomass on dry mater basis.

Nutrient	Concentration (g/100g)
Moisture	21.46 ± 1.50
Metabolizable Energy poultry (kcal/kg)	2465
Crude Protein	64.62 ± 1.04
Crude Fiber	0.50 ± 0.09
Ether Extract	0.170 ± 0.001
Nitrogen Free extract	6.64 ± 0.40
Salt1	0.194 ± 0.04
Ash	6.61 ± 0.33
Calcium1	0.22 ± 0.03
Phosphorus1	1.16 ± 0.06
Non-phytate phosphorus1	0.88
Sodium1	0.74 ± 0.08
Digestible Aspartamic acid	6.02 ± 0.30
Digestible Glutamic acid	8.86 ± 0.44
Digestible Serine	2.31 ± 0.12
Digestible Histidine	1.34 ± 0.07
Digestible Glicine	4.50 ± 0.22
Digestible Arginine	5.38 ± 0.27
Digestible Alanine	5.39 ± 0.27
Digestible Cistine	0.77 ± 0.04
Digestible Valine	5.36 ± 0.27
Digestible Methionine	2.07 ± 0.10
Digestible Phenilalanine	3.98 ± 0.20
Digestible Isoleucine	3.33 ± 0.17
Digestible Leucine	6.11 ± 0.31
Digestible Lysine	3.57 ± 0.18

[1]As part of the ash content.

and calculated as digestible according to Brazilian tables for birds and pigs, as well as metabolizable energy for birds (Rostagno *et al.*, 2017). The diets were isoproteic (18% crude protein) and isoenergetic (2850 kcal/kg) with four levels of inclusion of *Arthrospira maxima*: T0 (0% microalgae - control), T2 (2% microalgae), T4 (4% microalgae), and T6 (6% microalgae). T0 maintained 3 mg/kg of canthaxanthin in the formulation due to normal market requirements for egg yolk color, while the other treatments did not have synthetic egg yolk pigment. Table 2 shows the composition of the diets.

Table 2 – Ingredient composition of the experimental diets for laying hens, formulated using the software Brill® in as fed basis.1

Ingredient	T0 (%)	T2 (%)	T4 (%)	T6 (%)
Yellow corn	56.87	57.52	58.74	57.92
Soybean meal	26.50	24.06	20.80	19.15
Calcium carbonate (coarse)	6.00	6.00	6.00	6.00
Calcium carbonate (fine)	3.70	3.71	3.76	3.87
Wheat middlings	3.00	3.00	3.47	4.00
Arthrospira maxima	0.00	2.00	4.00	6.00
Soy oil	2.00	1.89	1.50	1.50
Monocalcium phosphate	0.69	0.62	0.54	0.46
Vitamin and Mineral premix	0.25	0.25	0.25	0.25
Salt	0.24	0.22	0.18	0.21
DL-Methionine	0.23	0.21	0.20	0.17
Sodium bicarbonate	0.20	0.18	0.19	0.14
Betaine	0.10	0.10	0.10	0.10
Micotoxin binder	0.10	0.10	0.10	0.10
Sodium butyrate	0.05	0.05	0.05	0.05
L-Lysine	0.04	0.05	0.08	0.06
Liver protector	0.03	0.02	0.03	0.03
L-Threonine	0.01	0.02	0.02	0.00
Canthaxanthin	0.003	0.00	0.00	0.00
Total	100	100	100	100
Nutritional composition1				
EM poultry kcal/kg2	2850	2850	2850	2850
Crude protein, %	18.63	18.81	18.74	18.91
Ether extract, %	4.40	4.29	3.92	3.89
Crude Fiber, %	2.51	2.43	2.39	2.37
Calcium %	4.28	4.27	4.27	4.31
Total Phosphorus, %	0.52	0.51	0.51	0.51
Digestible Phosphorus%	0.42	0.42	0.41	0.42

[1]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusion, T6%: *A. maxima* inclusion 1 formulated according to ISA Brown Nutrient Requirements (Hendrix Genetics, 2020b).

Evaluated variables and sample collection

The productive performance of the hens was measured weekly, including feed consumption, egg weight, egg mass, and feed conversion rate (FCR, kg feed/kg eggs). In addition, eggs were graded, and the proportion of A-type eggs was calculated weekly using the following equation:

% Type A Eggs= (Total eggs-(dirty eggs+pale egg+ broken eggs))/Total Eggs x 100

The egg quality was evaluated weekly using a sample of 15 eggs per treatment (3 eggs randomly selected from each replicate). Egg weight was determined using a precision balance (minimum detection of 0.1 g). Then, the egg content was placed on a flat surface, and the height of the dense albumen was measured using a caliper. Finally, the Haugh units were calculated using the following equation based on the egg weight (w, grams) and height of the dense albumen (h, millimeters):

UH=100 x log[(h-1.7 x w x 0.37+7.6)]

The yolk color was determined using the Digital Yolk Fan™ from DSM, and the eggshell thickness was measured using a digital caliper.

Additionally, nine eggs per treatment were randomly selected at the end of the experimental period to obtain a pooled sample for the analysis of fatty acid levels and cholesterol. The laboratory analysis was performed by the National Center for Food Science and Technology (CITA, for its acronym in Spanish) at the University of Costa Rica. The fatty acid profile was evaluated using AOAC 996.06 (AOAC 2005) and method Ce 1e-91, (AOCS, 2001) Gas Chromatography (GC-FID), P-SA-MQ-034. The cholesterol level was determined using high-pressure liquid chromatography (HPLC-PDA), developed according to the method described by Bauer *et al.* (2013).

Statistical Analysis

The variables were analyzed using statistical software R, version 3.5.1 (R Core Team 2018). Egg weight, egg mass, feed conversion ratio, shell thickness, and Haugh units were evaluated using mixed models of repeated measures, using the cage as the subject and the week as the time factor. Models for these variables with a normal distribution were adjusted using the complementary nlme package (Pinheiro *et al.*, 2020). For A-type eggs, a beta distribution was assumed for the error, modeled with the complementary glmmTMB package (Brooks *et al.*, 2017). Finally, an ordinal logistic model was used for egg yolk color, adjusted with the ordinal package (Christensen, 2019). After the described analysis, the Tukey test (p<0.05) was used to find differences between treatments. Since the concentration of egg fatty acids and cholesterol levels did not have a normal distribution, the variables were analyzed with a generalized beta linear model, with a logit function, using the betareg package (Cribari-Neto & Zeileis 2010).

Formulation Costs

The formulation cost of each experimental diet was determined using the value of each feed component and the estimated cost of microalgae production reported by TEC under experimental conditions.

RESULTS

Effect of *Arthrospira maxima* dietary inclusion on productive performance and egg composition

The result of the in vitro digestibility test for dry matter showed high digestibility (99.7 ± 0.01) g/100 g of dry matter with no microbial contamination. Table 3 shows the feed intake, and feed refusal was observed during the first week for laying hens fed the T 6% diet, probably due to lower palatability and adjustment to the new feed. The feed refusal did not show a negative effect on feed conversion ratio (FCR) and there was a tendency to increase feed conversion for the T6% treatment by 0.4 points with respect to the control at week 54, with no statistical difference (Figure 1, A).

Table 3 – Feed intake (g/day) of laying hen fed with different inclusion of levels of *Arthrospira maxima* in the diets, during the experimental period (52 to 55 weeks of age).[1]

Feed Intake (g/day)					
Treatment	Week 52	Week 53	Week 54	Week 55	Ave.
T0	123	118	123	126	123
T2	125	125	126	127	126
T4	127	125	125	125	125
T6	111	116	116	116	115

[1]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusion, T6: 6% *A. maxima* inclusion, Ave.: Average throughout the experimental period.

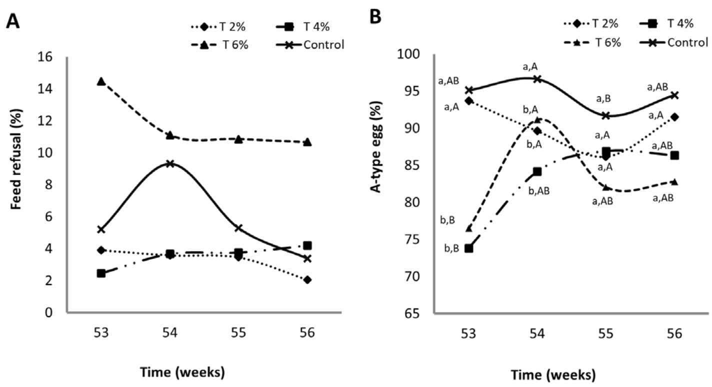

Figure 1 – Effect of the inclusion of *Arthrospira maxima* in laying hen diets on percentage of (A) Feed refusal and (B) A-type eggs during the experimental period (52 to 55 weeks of age). (B) Different lower-case letters in the same week indicate significant differences between treatments (*p*<0.05) weekly. Different capital letters in the same treatment indicate significant differences between weeks in the same treatment (*p*<0.05). T 0% (Control), T2%, T 4% and T 6% (the percentage refers to the inclusion of *A. maxima* in the diet).

The inclusion of *Arthrospira maxima* in the diets did not affect weekly egg weight, egg mass production, or feed conversion ratio (FCR). Tables 4 to 6 show the absence of significant differences between treatments or a time effec0t for those variables. A significant difference was observed for the average egg mass production during the 4-week trial period (Table 5) due to a numerical difference in egg production for the T6 group during the first 2 weeks of the experiment that created a significant difference for the total egg mass.

During the experimental period, a decrease in A-type eggs was observed for treatments T4 and T6 ($p<0.05$) (Figure 1, B). This was caused by an increase in dirty eggs for laying hens fed microalgae diets. The effect was due to green pigmentation of the eggshells caused by beak marks from laying hens after eating and drinking water, but diarrhea was not observed.

For egg composition, the results did not show differences in the concentration of total cholesterol levels due to the inclusion of *A. maxima* in the diets. The fatty acid profile indicated that the monounsaturated fatty acids (MUFA) were affected by the inclusion of *A. maxima* in the diets ($p<0.05$). T2 and T4 had the highest and lowest MUFA concentration per egg, respectively. The general results by treatment can be observed in Table 7, where an increase in fatty acids can be observed for T2, a decrease in T4, and again an increase for T6.

Table 4 – Effect of the inclusion of *Arthrospira maxima* in laying hen diets on the egg weight (g) throughout the experimental period. (52 to 55 weeks of age).[1]

Treatment	Estimated	Error St	df	t- value	*p*
T0	65.2699	0.6047	23.157	107.946	<2e-16***
T2	0.15	0.5459	69	0.275	0.784
T4	-0.3875	0.5459	69	-0.71	0.48
T6	-0.44	0.5459	69	-0.806	0.423
Week 53	0.0845	0.5459	69	0.155	0.877
Week 54	0.5155	0.5459	69	0.944	0.348
Week 55	0.6345	0.5459	69	1.162	0.249

[1]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusión, T6: 6% *A. maxima* inclusión. Ave.: Average throughout the experimental period.

Table 5 – Effect of the inclusion of *Arthrospira maxima* in laying hen diets on the egg mass (kg/week) during the experimental period (52 to 55 weeks of age).[1]

Treatment	Estimated	Error St	df	t- value	*p*
T0	2161.78	50.77	73	42.578	< 2e-16***
T2	-49.8	54.28	73	-0.918	0.3619
T4	-83.9	54.28	73	-1.546	0.12649
T6	-154	54.28	73	-2.837	0.00589
Week 53	25.05	54.28	73	0.462	0.6458
Week 54	50.75	54.28	73	0.935	0.35287
Week 55	-2.3	54.28	73	-0.042	0.96632

[1]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusión, T6: 6% *A. maxima* inclusión. Ave.: Average throughout the experimental period.

Table 6 – Effect of the inclusion of *Arthrospira maxima* in laying hen diets on feed conversion ratio (FCR, kg feed/kg eggs) during the experimental period (52 to 55 weeks of age).[1,2]

	week 1	week 2	week 3	week 4	lin	Cuad
T0	2,00(0,079)^{a,A}	1,91(0,079)^{a,A}	1,92(0,079)^{a,A}	2,09(0,079)^{a,A}	0,9986	0,5595
T2	2,05(0,079)^{a,A}	2,04(0,079)^{a,A}	2,05(0,079)^{a,A}	2,17(0,079)^{a,A}	0,5420	0,6736
T4	2,13(0,079)^{a,A}	2,08(0,079)^{a,A}	2,07(0,079)^{a,A}	2,14(0,079)^{a,A}	0,8584	0,5461
T6	1,99(0,079)^{a,A}	2,05(0,079)^{a,A}	2,00(0,079)^{a,A}	1,95(0,079)^{a,A}	0,5600	0,2561
lin	0,3379	0,1230	0,9948	0,7319		
cuad	0,0805	0,5571	0,5796	0,7403		

[1]Different lower-case letters in the same column indicate significant differences between treatments ($p<0.05$) weekly. Different capital letters in the same row indicate significant differences between weeks in the same treatment ($p<0.05$).

[2]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusion, T6: 6% *A. maxima* inclusion.

Effect of the dietary inclusion of *Arthrospira maxima* on selected egg quality traits.

The eggshell thickness ranged between 0.38 and 0.45 mm across treatments and trial duration, and a small decrease during the last week of the trial cannot be attributed to microalgae, but it seems to be associated to the egg size, the possible causes of these results will be addressed in the discussion section. Furthermore, there was no effect on Haugh units due to the treatments, the results were 80 or more throughout the trial.

Effects on egg yolk color were observed in this trial (Table 8), where a higher inclusion of microalgae increased the color according to DSM color scale, and a time effect was also observed.

Finally, the cost of experimental diets was compared to the control treatment (T0). Adding *Arthrospira maxima* meal to laying hen diets under experimental conditions increased costs per 1000 kg of feed by US$3.72, US$7.59, and US$13.8 for T2, T4, and T6, respectively. This increase is due to the substitution of

Table 7 – Effect of the dietary inclusion of *Arthrospira maxima* in the fatty acid profile (g/100g) and cholesterol level1 (mg/100 g) of chicken eggs.[1,2,3]

Fatty Acid (%)	T0	T2	T4	T6	Lin	Cuad
SFA	3,40 (0,121)a	3,58 (0,121)a	3,17 (0,121)a	3,41 (0,121)a	0,8757	0,9926
UFA	1,91 (0,071)a	1,93 (0,116)a	1,72 (0,082)a	1,84 (0,102)a	0,6616	0,9380
MUFA	4,58 (0,127)ab	4,98 (0,184)a	4,28 (0,135)b	4,49 (0,205)ab	0,4753	0,9236
n3	0,05 (0,003)a	0,06 (0,070)a	0,03 (0,031)a	0,06 (0,074)a	1,0000	0,9939
n6	1,79 (0,066)a	1,87 (0,086)a	1,68 (0,076)a	1,79 (0,108)a	0,9374	0,9969
n9	4,36 (0,121)a	4,67 (0,211)a	4,01 (0,161)a	4,21 (0,179)a	0,3096	0,9840
Colesterol1	160,0 (14,4)a	161,7(14,4)a	153,0 (14,4)a	180,3 (14,4)a	0,8229	0,7811

[1]Cholesterol expressed as mg/100g of egg yolk.

[2]Different lowercase letters represent significant differences ($p<0.05$) between treatments.

[3]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusión, T6: 6% *A. maxima* inclusión SFA-saturated fatty acids, PUFA-polyunsaturated fatty acids, MUFA-monounsaturated fatty acids, n3-omega 3 fatty acids, n6-omega 6 fatty acids, n9-omega 9 fatty acids, Chol.-Cholesterol level.

Table 8 – Effect of the dietary inclusion of *Arthrospira maxima* in laying hen feed on egg yolk color (using a Digital Yolk Fan™ of DSM).

	week 1	week 2	week 3	week 4	lin	cuad
T0	13,60(0,132)a,A	13,14(0,129)a,AB	12,79(0,131)a,B	12,28(0,136)a,C	0,0077	0,0000
T2	12,26(0,130)c,A	11,13(0,133)c,B	10,05(0,145)d,C	9,65(0,162)d,C	0,0000	0,0000
T4	12,72(0,137)bc,A	11,86(0,122)b,B	11,26(0,137)c,C	11,00(0,131)c,C	0,2297	0,0000
T6	12,87(0,120)b,A	12,07(0,116)b,B	12,07(0,115)b,B	11,53(0,142)b,C	0,3858	0,0000
lin	0,0000	0,0000	0,0000	0,0000		
cuad	0,9950	0,0310	0,0598	0,6122		

[1]Different lower-case letters in the same column indicate significant differences between treatments ($p<0.05$) Different capital letters in the same row indicate significant differences between weeks in the same treatment ($p<0.05$).

[2]T0: 0% *A. maxima* inclusion, T2: 2% *A. maxima* inclusion, T4: 4% *A. maxima* inclusion, T6: 6% *A. maxima* inclusion.

soybean meal (US$ 0.39/kg) with microalgae in the diet (US$ 0.79/kg). It was also observed that savings generated by eliminating the addition of canthaxanthin as an artificial pigment due to the incorporation of microalgae did not offset the costs compared to the control treatment (T0).

DISCUSSION

The qualitative analysis of *A. maxima* biomass demonstrated that it is safe for use as a feed additive when cultured in wastewater. As recently reported, *Arthospira* sp. tends to take up macronutrients and micronutrients from wastewater and has shown less absorption affinity for heavy metals (Cardoso *et al.*, 2020). The digestibility test indicated high nutritional availability of the dry biomass, which complements the low crude fiber content, which usually is not metabolized. The microalgae culture production yield agreed with previous reports in raceway systems for Arthrospira sp. (Grahl *et al.*, 2018; Purohit *et al.*, 2019). Nutrient and pH control in the medium allowed for continuous culture and extended logarithmic phase, avoiding the stationary phase of growth and ammonium toxicity that cause productivity reduction (Lu *et al.*, 2019).

Effect of *Arthrospira maxima* dietary inclusion on productive performance and egg composition

The egg production results of this trial are comparable to the ISA Brown standards for hen age (ranging from 91.3 to 89.8%; Hendrix-Genetics 2020a) and partially agree with Omri *et al.* (2019), who determined that there is no significant difference in egg production when 1.5% and 2.5% *Arthrospira maxima* is added to the diets of 44-week-old Lohmann laying hens. They also did not find a time effect during the 6-week experimental period. In addition, Zahroojian, *et al.* (2013) did not report any differences in the productive performance of 63-week-old Hy-line W36 laying hens that were supplemented with 1.5%, 2.0%, and 2.5% *Arthrospira maxima* during a 3.5-week experimental period. Our results indicate a lower overall egg mass for the T6 treatment group, which was likely affected by lower feed intake during the first two weeks of the trial. To our knowledge, there are no reports in the literature indicating a 6% Arthrospira sp. inclusion in the diet, and these results could indicate that laying hens need an adaptation period before they can reach their normal production performance.

On the other hand, different results are reported with laying hens where an increase of 4.99% in egg production was observed with the supplementation of 3% *Arthrospira maxima* in substitution of wheat bran during a 25-day trial (Parra *et al.*, 2017). However, in that trial, the Arthrospira diet had 3.53% more protein and 8.59% more fat, giving the laying hens a nutritional advantage over the control.

Although there was a reduction in soybean meal in the diets, egg weight was not affected mainly due to the contributions of lysine and methionine from *Arthrospira maxima*, as reported previously (Alvarenga *et al.*, 2011), both limiting amino acids for egg weight (Salvador & Guevara, 2013). Other results indicated an increase in egg weight of 0.42 g and 1.57 g supplemented with 1.5% and 2.5% *A. platensis*, respectively, becoming higher in crude protein content, compared to the control (Omri *et al.*, 2019).

Other authors found results that disagreed with the present study. Selim *et al.* (2018) did not find any differences in egg production and egg mass in an 8-week supplementation trial with 38-46-week-old laying hens. However, during the last 4 weeks of their experiment, they observed an increase in both parameters, so the differences in the overall averages of our research could be due to their short experimental period and the fact that the laying hens need more time to adjust to diets with higher levels of inclusion to *Arthrospira maxima*. It is important to mention that the egg weight and egg mass obtained in this experiment for all treatments are higher than the ISA Brown standards for age (Hendrix-Genetics, 2020a). In Costa Rica, eggs are sold by the kilogram, so for producers, not only egg weight but also the total mass of eggs produced per batch is important.

A trend towards lower egg production was observed during the first and second week of the trial for T6, which could have been caused by a decrease in feed intake. The lower feed intake for T6 could be due to a lower palatability of the diet with a higher level of *Arthrospira maxima*. This behavior has not been reported in other literature for laying hens, however, as mentioned before, there are no other trials with such a high level of inclusion as 6%, as in the present study. Nguedia *et al.* (2019) reported a decrease of 6.13 g/animal/day in dry matter intake for guinea pigs supplemented with 6% *Arthrospira maxima* compared to supplementation at 4%.

However, the lower feed intake did not result in a change in feed conversion ratio (FCR). The absence of differences in FCR indicates that the substitution of soybean meal as a protein source apparently did not affect the digestibility or utilization of nutrients due to its high biological value (Shimamatsu, 2004; Alvarenga *et al.*, 2011; Batista *et al.*, 2013; Holman & Malau-Aduli, 2013; Sprujit *et al.*, 2016). However, due to the nature of the commercial farm where this trial was conducted, there was no opportunity to weigh the laying hens during the experimental period.

The increase in second-grade eggs was due to the presence of dirtier eggs. The experimental diets turned green due to the inclusion of microalgae; the more microalgae included, the greener the mash became, caused by the dissolution of chlorophylls and phycocyanin highly concentrated in *A. maxima*. This caused a green liquid to drip from the beaks of the laying hens onto some of the eggs when they were laid. The green spotted eggs do not represent any risk for human consumption, due to the innocuity of the pigments.

The results of the total cholesterol level in this trial are contrary to the findings of Selim *et al.* (2018) who reported that supplementation of 0.3% *A. platensis* led to a decrease in cholesterol content levels of eggs from 13.6 to 11.7 mg/g due to a decrease in serum triglyceride and cholesterol levels from 135 to 115 mg/dl. In addition, Mariey *et al.* (2012) determined that supplementation of 0.2% *A. platensis* resulted in a reduction of 23.83 mg/100 ml of triglycerides, 7.34 mg/ml of plasma cholesterol level, 59.6 mg/g of total lipids, and 3.3 mg/g of cholesterol level in the egg yolk compared to a corn and soybean meal diet.

As shown in Table 7, only MUFA showed statistical differences. However, the differences do not show a trend according to the level of microalgae inclusion. This is due to the low ether extract (EE) content of the microalgae (Table 1), which does not have much participation in the fatty acid profile.

The deposition of fatty acids in the egg yolk is directly related to the content and composition of the ether extract (EE) of the diets offered to laying hens (Griffin & Hermier, 1988; Sartori *et al.*, 2009; Cherian, 2015).

An increase in the yield of fatty acids in microalgae biomass produced in wastewater has been reported, which differs from the results of this study, where a lower lipid yield was obtained in relation to autotrophic cultures. The average fatty acid profile has been reported for this microalga, and it is mainly rich in palmitic acid and palmitoleic acid, each accounting for up to 40%, and oleic acid and linoleic acid with percentages ranging from 9 to 15%, as found in other feed matrices such as corn and soy. Other important lipids in *A. maxima* are omega-3 fatty acids such as linoleic acid and cis-8,11,14-eicosatrienoic acid and would represent a contribution to the fatty acid profile of the egg if the percentages in the biomass were higher than those found in the biomass used in this trial (Cañizares-Villanueva *et al.*, 1995; Borges *et al.*, 2013).

Finally, the drying method and storage time may have influenced the lipid profile of the microalgae meal. Long times of storage of microalgae biomass has shown oxidation of the fatty acids and a decrease in antioxidants. Nouri & Abbasi (2018) found a 20% reduction in antioxidant activity in sun-dried *Arthrospira maxima* biomass compared to sealed vacuum oven drying. On the other hand, storage time and temperature have a negative effect on the antioxidant activity of *Arthrospira maxima* biomass (Colla *et al.*, 2016). The inhibition on the functional effect expected for this trial, is attributed mainly in reducing cholesterol levels in eggs and increasing omega-6 fatty acids in eggs (Romay *et al.*, 2003; Eriksen, 2008; Mariey *et al.*, 2012; Cuellar-Bermudez *et al.*, 2015; Selim *et al.*, 2018). In addition to lipid content, other important aspects of *A. maxima* cultivation, such as the ability to grow to reuse water, feasible biomass recovery from the culture medium, and high protein content, should be considered when choosing a microalga for animal feed.

Effect of the dietary inclusion of *Arthrospira maxima* on selected egg quality traits

The statistical analysis does not show a clear effect of treatments on shell thickness. However, a decrease can be observed for all treatments starting from the second week. Thinner eggshells may be related to the age of the hens, as it has been determined that eggshell quality decreases during the later egg production period (55 to 65 weeks) (Roberts *et al.*, 2013). This phenomenon is related to the laying hen's egg formation physiology, where the eggshell deposit remains the same throughout the production period, but the size of the egg increases over time, diluting the amount of shell and affecting its thickness. In addition, high ambient temperatures affect eggshell quality if hens begin to pant as a mechanism for evaporative cooling, causing an imbalance in blood pH that prevents calcium carbonate deposition in the eggshell (Allahverdi *et al.*, 2013). Although temperature was not measured during this trial, the province of Alajuela has very hot days during the year, which could affect egg quality.

No effects were observed on the Haugh units in this trial due to the use of microalgae. It is important to mention that this quality indicator can be affected by other conditions such as laying hen age, feed formulation and management, environmental and egg storage temperature, egg age, and other non-dietary factors (Hy Line International, 2017).

Regarding egg yolk color, the addition of *Arthrospira maxima* affected the results, indicating that algae have pigmenting capacity despite a small numerical decrease in feed intake. However, a 6% inclusion of the microalgae was necessary to achieve the market color accepted by Costa Ricans (12 on the DSM scale) with a yolk color vary from yellow to orange, according to The Central American Technical Regulation RTCR 397:2006 (Ministerio de Agricultura y Ganadería, 2006). An effect due to time was also observed, in the case of the control diet (T0), there was a slight decrease in feed intake over the weeks, which could have affected pigment deposition. In the case of the experimental diets, it is necessary to investigate if the diets prior to the trial have a dragging effect on pigment deposition that dissipates over time. A longer observation period is recommended to evaluate if the color continues to decrease. Additionally, an effect of the drying process and storage time of the algae was addressed to explain the results in the fatty acid profile, and the same effects could be contributing to a lower pigmenting capacity of the microalgae due to oxidation.

These results are contrary to those reported by Zahroojian *et al.* (2013), who determined that an inclusion of 2.5% freeze-dried *Arthrospira maxima* in diets without artificial colorants was sufficient to obtain a color score of 11.66 on the DSM scale, which means that a lower concentration of the microalgae was needed to achieve a high color score. These differences may be related to the effect of algae processing (freeze-drying extrusion) on the preservation of carotenoid pigments, where freeze-drying better preserves the components of microalgae and adds more color to the yolk (Anderson *et al.*, 1991; Ross *et al.*, 1994). In addition, it has been reported that the convection drying used to obtain the microalgae used in that trial decreases the effect of *Arthrospira maxima* on egg yolk pigmentation (Parra *et al.*, 2017).

All treatments showed a decrease in egg yolk color in the time, which could indicate that there could be a residual effect of the control diet provided before the trial. A longer experimental period is needed to explore these results more deeply, but the results obtained were promissory.

In conclusion, the addition of *Arthrospira maxima* in diets of 52-week-old laying hens at levels of 2% and 4% did not affect the productive performance of the laying hens during a 4-week trial period (egg weight, egg mass, FCR, eggshell thickness, and Haugh units). The inclusion of microalgae at a level of 6% produced an acceptable egg yolk color on the DSM scale without artificial colorants in the feed, but a decrease in feed intake at the beginning of the trial created lower egg production and egg mass overall, suggesting an adaptation period for the hens to eat the higher inclusion level diet.

The use of *Arthrospira maxima* did not affect the cholesterol level or fatty acid concentration of the eggs. More information is needed to determine if the drying or storage process influenced these results.

The inclusion of *Arthrospira maxima* in diets increased the formulation costs under experimental conditions, so for the feed to be competitive in a lower cost feed formulation software, its cost should not be higher than the sum of the usual protein source plus the artificial colorant.

ACKNOWLEDGMENTS

The authors would like to thank Granja Avícola Los Pollitos for allowing the use of their chickens during this experiment, Vetim S.A. for mixing the diets used during the trial, and Luis Alejandro Rodríguez-Campos for his support in statistical analysis.

FUNDING

This research was funded by the Technological Institute of Costa Rica (TEC) through project No. 1510105 “Protein and lipid characterization of biomass obtained from microalgae Isochrysis galbana and *Arthrospira maxima* as a potential source of functional food” and the University of Costa Rica (UCR) through project No. 739-B8-209 “Use of microalgae in the feeding of poultry and pigs”.

CONFLICTS OF INTEREST

None

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