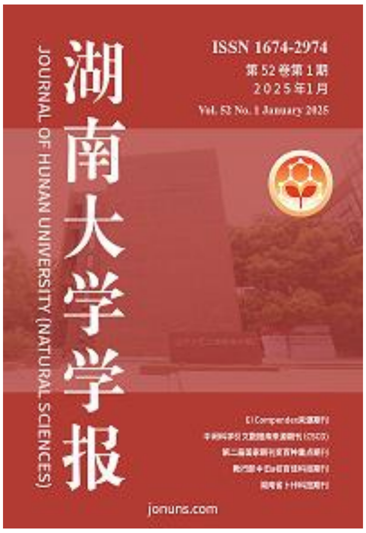

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Eggs with Higher Antioxidant Content Caused by the Biotechnological Development of Microalgae in Cage-Free Hens

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Abstract: This study aimed to examine the effects of supplementation with the microalgae *Arthrospira* (*Spirulina*) *platensis* and *Haematococcus pluvialis* on the production and quality of eggs from Hy-Line Brown laying hens under heat stress conditions. The research was conducted at the Canchones campus of Arturo Prat University, located in the Atacama Desert, Chile. A total of 125 hens aged 22 to 36 weeks were used in an experimental design with five treatments: a) a control group without microalgae, b) groups with 1% spirulina and 20, 40, or 60 ppm of natural astaxanthin from *Haematococcus pluvialis*, and c) a group with 1% spirulina. The results showed that microalgae supplementation significantly improved egg production and live weight gain in the laying hens, particularly under heat stress conditions. Higher levels of dietary antioxidants correlated with better performance, surpassing the control group and industry benchmarks. Yolk color, which was strongly correlated with carotenoid content, increased significantly with higher levels of microalgae supplementation. The group with the highest concentrations of spirulina and astaxanthin produced the most intense yolk color and the highest carotenoid content. This study highlights the potential of microalgae supplementation to improve egg production and quality in cage-free laying hens, especially in environments with heat stress.

Keywords: Microalgae, antioxidants, heat stress, egg quality, egg production

通过开发无笼养母鸡的微藻生物技术，生产出抗氧化剂含量更高的鸡蛋

摘要： 本研究旨在考察在热应激条件下补充微藻螺旋藻和雨生红球藻对海兰褐产蛋母鸡的产蛋量和鸡蛋品质的影响。这项研究是在位于智利阿塔卡马沙漠的阿图罗普拉特大学 Canchones 校区进行的。实验设计共使用 125 只龄为 22 至 36 周的母鸡，设置五种处理方法：a) 不含微藻的对照组，b) 含 1% 螺旋藻和 20、40 或 60 ppm 雨生红球藻天然虾青素的组，以及 c) 含 1% 螺旋藻的组。结果表明，补充微藻显著提高产蛋母鸡的产蛋量和活重增加，尤其是在热应激条件下。膳食抗氧化剂含量越高，生产性能越好，超过了对照组和行业基准。蛋黄颜色与类胡萝卜素含量密切相关，随着微藻补充水平的提高，蛋黄颜色显著增加。螺旋藻和虾青素浓度最高的组蛋黄颜色最深，类胡萝卜素含量最高。这项研究强调了微藻补充对提高无笼产蛋母鸡产蛋量和产蛋质量的潜力，尤其是在热应激环境中。

关键词：微藻、抗氧化剂、热应激、蛋品质、蛋产量

1. Introduction

Industrially produced eggs, with high biological value, provide proteins rich in essential amino acids. This could promote the synthesis and maintenance of human skeletal muscle mass. This property may be particularly relevant for athletes and older adults, helping the latter counteract the process of sarcopenia, which is typical of aging. The main protein in egg white is ovalbumin, followed by ovotransferrin and lysozyme [1]. Egg proteins may be beneficial against inflammation and possess antimicrobial, immunoprotective, antihypertensive, and antioxidant properties [2]. Furthermore, the yolk contains immunoglobulin (IgY), the main avian serum antibody, which is functionally equivalent to mammalian immunoglobulin G. Both in vitro and in vivo, IgY inhibits the development of infections by gastrointestinal pathogens such as rotavirus, *Escherichia coli*, and others [3]. It has been shown that the lipids and phospholipids present in the yolk have antioxidant effects and prevent the oxidation of unsaturated fatty acids. A phospholipid, such as phosphatidylcholine, are an important source of choline, an important nutrient for brain development, liver function, and cancer prevention. Phospholipids (PL) with special biological activity are used to treat chronic diseases such as cardiovascular and cerebrovascular diseases [3]. Eggs are also one of the main sources of vitamin D in the diet and provide many other nutrients such as riboflavin, folate, selenium, vitamin A, and vitamin B12, among others [1]. Some of these nutrients (such as zinc, selenium, retinol, and tocopherols) are often deficient in people who eat a Western diet. Given the antioxidant capacity of these nutrients, they are potential protectors against cardiovascular diseases [1].

Another relevant characteristic of eggs is their contribution of carotenoids to the human diet, such as lutein and zeaxanthin, which have antioxidant properties that protect against cataracts and macular degeneration, important causes of blindness in old age [4]. The addition of lutein significantly increases sperm viability, suggesting a potential influence on male reproductive parameters [5]. Synthetic carotenoids have been used since the 1990s in European countries to meet the requirements of consumers who prefer eggs with colored yolks [6]. An alternative is to produce eggs with natural antioxidants, where it is possible to add 1-2% microalgae to the food, allowing natural carotenoids to be incorporated into the yolk [6]. Lutein and zeaxanthin possess antioxidant and immunomodulatory properties, contributing to a stronger immune system in birds [7]. Supplementation in the laying hens' diet contributes to improving the intestinal microflora and, therefore, may play a significant role in improving the birds' health. It has been shown that the addition of *Chlorella vulgaris* to the diets of laying hens increases microbial diversity in the digestive tract, especially in the cecum [8].

The effects of *Haematococcus pluvialis* natural astaxanthin (ASTA) on production performance, egg quality, antioxidant enzyme activity, free radical scavenging capacity, and gene expression of antioxidant enzymes in laying hens were evaluated in [13]. The results demonstrate that dietary ASTA improves the free radical scavenging capacity and antioxidant enzyme activity. It also reduced the plasma levels of low-density lipoprotein cholesterol and triglycerides [10]. Similar to spirulina, the addition of Astaxanthin (*Haematococcus pluvialis*) to the diet of broilers led to an improvement in the immunological characteristics of broilers reared under normal and elevated ambient temperatures [11]. Astaxanthin from *H. pluvialis* does not affect the quality of fresh eggs but rather extends the storage time of eggs [12]. *Spirulina platensis* in the Japanese quail had positive effects on egg quality. This is because it reduced the levels of saturated fatty acids, which are undesirable, and increased the levels of monounsaturated fatty acids, which are beneficial for consumer health. Finally, antioxidants were increased in the egg yolks [13].

This study aimed to evaluate the effect of supplementation with *Spirulina platensis* and *Haematococcus pluvialis* on egg production, weight gain and egg quality in Hy-Line Brown laying hens, particularly under heat-stress conditions.

2. Materials and Methods

2.1. Egg Quality

The study was conducted at the Avian Pathology Laboratory, University of Chile. Egg quality was assessed using several techniques. The eggshell strength was evaluated by measuring the thickness in three different zones and weight using an EggForce® machine. The procedure consisted of

i) Egg cracking: The eggs were introduced into the machine through a conveyor belt or hopper. A cracking mechanism, often involving rollers, gently cracks the eggshells.

ii) Separation: After cracking, the contents of the egg pass through a separation system. This is based on the difference in density between the egg white (albumin) and the yolk. This separation is achieved by centrifugal force: The egg mixture can rotate rapidly, causing the denser yolk to move outward or to the center, allowing for separation.

iii) Collection: Separated egg whites and yolks are collected in separate containers or holding tanks.

iv) Cleaning: The EggForce® machine is designed with hygiene in mind. It typically incorporates cleaning features to minimize contamination and maintain sanitary conditions during operation.

The egg weight was also recorded. Yolk color was determined using a DSM Yolk Color Fan (formerly Roche Yolk Color Fan), a 16-scale color index widely used in the poultry industry, and analyzed with an EggAnalizer® machine. This apparatus employs optical techniques, specifically transmittance/reflectance spectroscopy. The process involves illuminating the egg and measuring the transmitted and reflected light intensities. This enables the electronic measurement and calculation of egg weight, albumen height, yolk color, Haugh unit (a quantitative indicator of egg freshness), and United States Department of Agriculture (USDA) grade.

2.2. Experimental Design of the Diets

The study was conducted at the Canchones campus of Arturo Prat University, located in the Atacama Desert, Chile (20°16'19.6" S, 70°07'34.0" W). One hundred and fifty 22–36-week-old Hy-Line Brown hens were used. Spirulina microalgae (1%; supplied by Solarium Biotechnology S.A., grown in a raceway system and natural astaxanthin (20, 40, or 60 ppm); from *Haematococcus pluvialis*, supplied by Atacama Bio Natural Product S.A. were incorporated into five different diets (**Ошибка! Источник ссылки не найден.**). Hens were randomly assigned to five cage-free pens (five replicates per treatment), each measuring 6 L × 3 W × 2 m H, resulting in a stocking density of 0.72 hens/m². The ambient temperature ranged from 8 ± 0.1°C to 32 ± 0.1°C under a 16L:8D photoperiod (**Ошибка! Источник ссылки не найден.**).

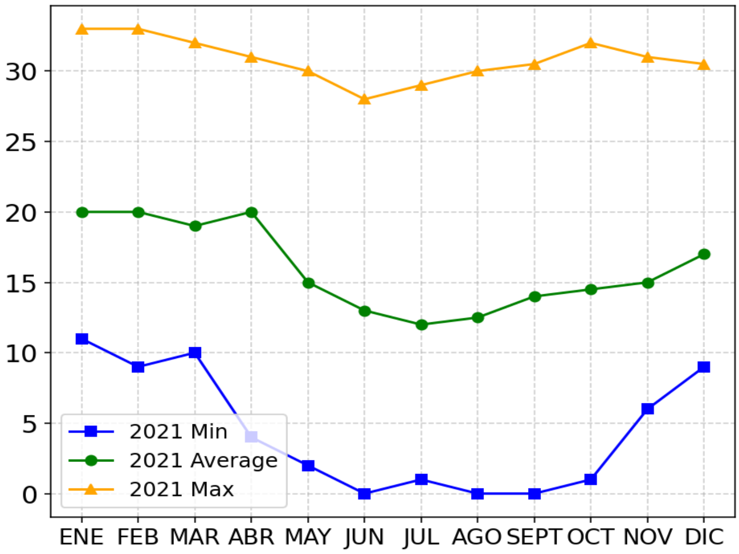


Figure 1. Temperature of the Atacama Desert, Tamarugal Pampa experimental area (developed by the authors)

Table 1. Experiment design (compiled by the authors

	Treatment 1 (T1)	**Treatment 2 (T2)**	**Treatment 3 (T3)**	**Treatment 4 (T4)**	**Treatment 5 (T5)**
Use of microalgae	Control Group: no microalgae	1% spirulina and 20 ppm ATS	1% spirulina and 40 ppm ATS	1% spirulina and 60 ppm ATS	1% spirulina
	T1	T 2	T3	T 4	T5
Repetition 1	5	5	5	5	5
Repetition 2	5	5	5	5	5
Repetition 3	5	5	5	5	5
Repetition 4	5	5	5	5	5
Repetition 5	5	5	5	5	5
Total birds in Test	**125**				

The basal diet (Table **2**) consisted of corn, dehulled soybean meal, and corn gluten meal, formulated according to Hy-Line Brown layer management recommendations for hens aged 22–36 weeks. Water was provided ad libitum.

Table 2. Ingredient composition of experimental diets (compiled by the authors)

Ingredients	**Content % (22 to 36 wk)**
Corn	64
Soybean meal	20
Corn gluten meal	0.38
DL-Methionine	0.35
Lysine HCL	0.24
Fat animal vegetable blend	2.75
Limestone	9.76
Mono-Dical PO4	1.66
Salt	0.29
Sodium bicarbonate	0.12
Vitamins1	0.27

Calculated nutrient composition (%)	**Content % (22 to 36 wk)**
E. Metabolize (Kcal/kg)	2911
Crude protein	15
Calcium	0.94
Phosphorous	0.43
Sodium	0.17

Note: Vitamin premix added at this rate yields (per kg): vitamin A, 8.8 IU; vitamin D3, 3.3 IU; vitamin E, 16 IU; vitamin K3, 2.2 mg; vitamin B12, 0.022 mg; vitamin K3, 2.2 mg; thiamine (B1), 1.7 mg; riboflavin (B2), 5.5 mg; niacin (B3), 28 mg; pantothenic acid (B5), 6.6 mg; pyridoxine (B6), 3.3 mg; Mn, 88 mg; Zn, 88 mg; I, 1.7 mg; Cu, 5.5 mg; Fe, 55 mg.

2.3. Determination of the Live Body Weight Gain

To determine the live body weight gain of birds LBWG (Kg/bird) during the study period. The following formula was used LBWG22-36 = LBWG36– LBWG22. The growth rate GR was calculated as follows:

$GR22\text{-}36=(LBW36–LBW22)/0.5(LBW22+LBW36)$.

The pullets were randomly marked for identification, and their data record was observed from 22 to 36 weeks.

2.4. Estimation of Total Carotenes in Egg Yolk

The pure liquid yolk was mixed with distilled water at a 1:1.5 (v/v) ratio and the solution was adjusted to pH 7.0 using 0.1 M NaOH. It was then centrifuged at 12,000 rpm for 45 minutes at 4° C, resulting in granules and plasma, which were freeze-dried separately. Carotenoid extraction was performed using commercial sunflower oil [14, 15] were quantified using a UV-Visible spectrophotometer (Hanna Iris-HI801, United States) at the wavelength of 450 nm [16]. From the absorbance values obtained in the spectrophotometric measurement, a standard curve of ß-Carotenes (Sigma Aldrich, 22040-1G-F) in sunflower oil [15].The methodology for the total carotenoid extraction involved two centrifugation steps. Samples were first heated to 55 °C in a water bath and then centrifuged at 12,500 rpm for 15 min to separate the granules and plasma. Carotenoid extraction from each fraction (3 g granules or plasma) was performed using 27 g of preheated (55 °C) sunflower oil. Following centrifugation at 13,000 rpm for 7 min, the supernatant was collected and analyzed. The carotenoids were quantified spectrophotometrically by measuring the absorbance of the supernatant at 450 nm, with sunflower oil serving as a blank. To determine the concentration of the total carotenoids extracted from the absorbance values obtained, a standard curve of β-carotene (Sigma Aldrich, 22040-1G-F) was created. The concentrations used ranged from 0 to 33 µg/mL, whose absorbance was measured at 450 nm as well as the extracted samples. By substituting the absorbance values into the equation derived from the linear regression and accounting for the performed dilutions, the µg of carotenoids per gram of sample (plasma or granules) were calculated.

2.5. Effectiveness of the Microalgae on the Immune Response of Laying Hens

To evaluate the efficacy of the microalgae on the immune response of laying hens, the birds were immunized by applying the vaccine against the Newcastle disease virus. Serum antibody titers (Immunoglobulin M and G) were measured using the ELISA (IDEXX, USA) technique, performed in the Avian Pathology Laboratory at the University of Chile.

2.6. Statistical Analysis

The pen served as the experimental unit for production performance assessments, while the number of birds per pen was the experimental unit for all other analyses. Data are expressed as mean ± SEM. Individual bird data were analyzed using one-way ANOVA (RStudio® 64-bit, Version 4.2.2). Statistical significance was defined as $P < 0.05$.

3. Results and Discussion

3.1. Weekly Hen Egg Production

In the present study, the use of microalgae biomass as a potential supplement in animal feed was investigated, focusing on diets for poultry that included various proportions of Spirulina and astaxanthin extracts from *H. pluvialis*. Experiments were conducted with four different treatment groups. The results of egg production are shown in Figure 2. According to the management manual for commercial laying hens Hy-Line Brown, production in cage systems reached an average of 92% at week 35. In this study, Treatment 4 (T4) achieved an egg production rate of 84% ± 0.05 under non-caged and free-range conditions. However, as reported in [17], Hy-Line Brown layers fed a soybean-based diet in free-range conditions, which resembled the conditions of the control group T1 in this study demonstrated 94% production rate. In comparison, T1 exhibited a significantly lower production rate of 58% ± 0.05.

The negative impact on production is correlated with thermal stress, which induces oxidative stress in the birds. However, it has been demonstrated that the inclusion of high levels of bioactive antioxidant compounds in the diets of layers can mitigate the damages caused by this oxidative stress, especially under elevated environmental temperatures, as evidenced by the results for treatment T4, corroborated by [18-20]. These findings suggest that the inclusion of microalgae biomass in poultry diets may have beneficial effects on egg production, particularly under thermal stress conditions (**Ошибка! Источник ссылки не найден.**).

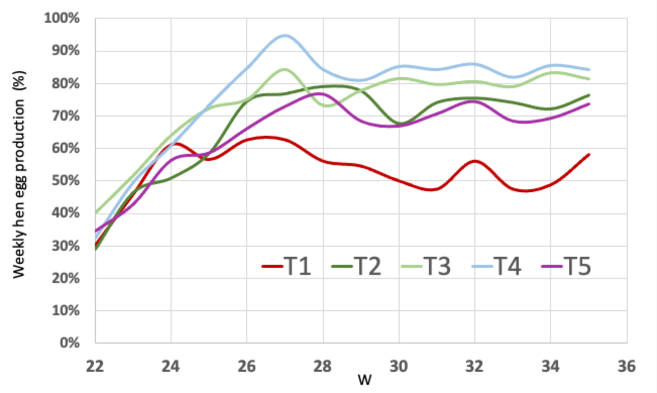


Figure 2. Weekly hen egg production: Treatment 1 (T1 control group, no microalgae), T2, T3, and T4 (Spirulina and natural astaxanthin of *Haematococcus pluvialis)* and T5 (only *Spirulina)* (developed by the authors)

The effects of antioxidants on poultry health and production were also reported [21]. Oxidative stress (OS) is a major concern that impacts overall poultry health in modern production systems. It is characterized by an imbalance between the antioxidant defense mechanisms and the production of reactive oxygen species (ROS) [22-24].

The notable difference in the percentage of egg production between treatments T4 and T1 at week 35 ($p < 0.05$) can be explained by T4's superior ability to adapt to thermal stress, as this treatment has a higher concentration of antioxidants (ATS) incorporated into the feed (Figure 3).

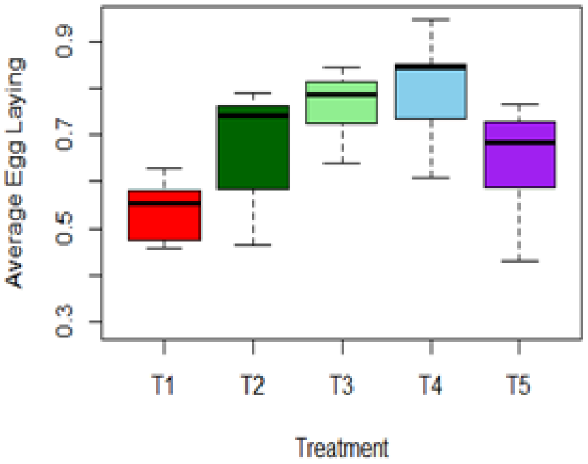


Figure 3. Percentage of egg production in week 35: Treatment 1 (T1 control group, no microalgae), T2, T3, and T4 (Spirulina and natural astaxanthin of *Haematococcus pluvialis*) and T5 (only *Spirulina)* Live weight total gain (developed by the authors)

According to [25], the incorporation of antioxidants in the diet of quails improves both egg quality and production. Treatments T1, T2, and T3 showed significant differences in egg production compared with the control group T1 ($p < 0.05$). However, the peak production was achieved in weeks 27 and 28, with average productivity values of 95% ± 0.02 for T4 (week 27), 84% ± 0.02 for T3 (week 27), 79% ± 0.02 for T2 (week 28), 77% ± 0.02 for T5 (week 28), and 63% ± 0.06 for T1 (week 27). This peak is consistent with the guidance provided in the Hy-Line Brown layer management manual, which states that optimal egg production occurs between weeks 27 and 30, with an average production of 94% per bird, significantly higher than the results observed in treatments T1, T2, T3, T4, and T5. Similarly, as reported in [17], 95% egg production was reached during week 27 in free-range diets.

3.2. Live Weight Gain

The evolution of live weight gain in laying hens between weeks 22 and 27 was not statistically significant ($p > 0.5$). However, notable differences in weight gain were observed between weeks 29 and 36 ($p < 0.05$). By week 36, the average weights recorded for the layers were as follows: T4 at 2.21 kg ± 0.04, T3 at 2.10 kg ± 0.04, T2 at 2.10 kg ± 0.04, T5 at 1.99 kg ± 0.04, and T1 at 1.88 kg ± 0.04 (Figure 4).

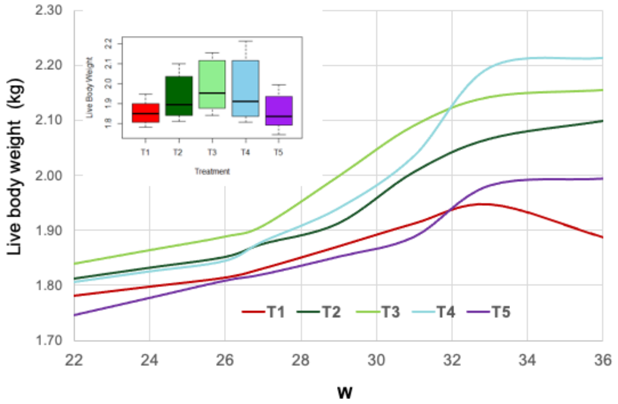


Fig. 4. Live weight total gain (Kg) between the different treatments and the control group (developed by the authors)

According to the Hy-Line Brown management manual, the expected body weight (W36) is 1.92 kg; treatments T2, T3, T4, and T5 all exceed this benchmark. Furthermore, it is noteworthy that the T1 control group experienced a weight loss from weeks 34 to 36 ($p < 0.05$), decreasing from 1.95 kg ± 0.04to 1.88 kg ± 0.02. This weight reduction parallels the trends observed in egg production, which is likely attributable to thermal stress. Notably, the incorporation of microalgae into the diets of poultry raised for egg and meat production increases body weight [26-28]. The inclusion of microalgae in broiler diets increases body weight and feed intake. This effect is likely attributable to the enhanced nutritional profile of the resulting poultry products, characterized by increased concentrations of omega-3 fatty acids and carotenoids.

3.3. Growth Rate between the Different Treatments

Regarding the growth rates, no differences were observed until week 31 (GR 22-31) ($p > 0.05$). However, after week 36, significant differences in growth rates were recorded between treatments ($p < 0.05$), with average values of GR T4 0.05 ± 0.03; T3 0.04 ± 0.03; T2 0.036 ± 0.03; T5 0.03 ± 0.03; and T1 0.01 ± 0.04. During the study period, all treatments experienced weight gain, except for the control group (T1), which achieved growth until week 32, after which it began to decline (**Ошибка! Источник ссылки не найден.**). Treatments with microalgae, such as *Haematococcus pluvialis* and Spirulina, showed improvements in growth factors in poultry intended for the egg. In this context, treatments T2, T3, T4, and T5 exhibited higher growth rates than the control group T1 ($p < 0.05$). These growth rate results are consistent with previous reports indicating that diets containing higher amounts of astaxanthin achieve higher growth rates, while T5, which contains only 1% Spirulina, remains in the last position. Consequently, there is evidence that the inclusion of microalgae such as *Chlorella* and *Spirulina* in the diet improves growth factors in birds (Fig. **5**) [17, 18, 26, 28-30].

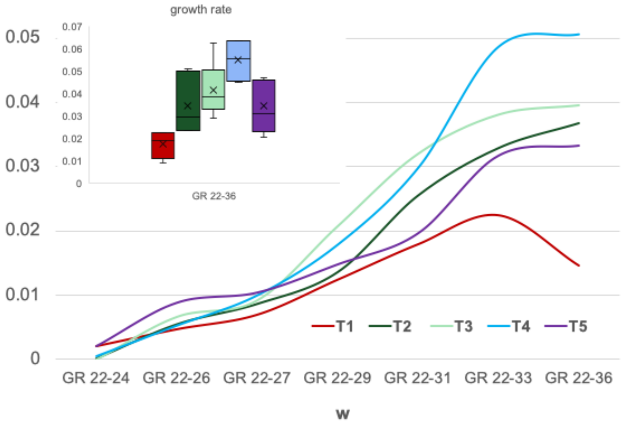


Fig. 5. Growth rate between the different treatments and the control group (The authors).

Microalgae are regarded as factories for microcellular nutrient reserves, and when used as feed ingredients, they tend to have a positive influence on the physics characteristics of hens, such as body weight gain, as evidenced in this case with treatments T2, T3, T4, and T5, though to a lesser extent T5 [31, 32]. To date, no studies have been reported where two microalgae (*Spirulina* and *Haematococcus pluvialis*) are incorporated into the birds' feed, as in this study.

3.4. Egg Quality at Week 35

The egg weight and eggshell resistance variables, which measure the quality of the egg until week 35, did not present a difference between the different treatments ($p > 0.05$), showing the following average weights for the different treatments: T4 60.5 g ± 0.09; T2 59.4 g ± 0.9; T3 59.3 g ± 0.9; T5 58.3 g ± 0.9 and T1 57.3 g ± 0.9 (Figure 6A). According to the Hy-Line Brown management manual, the expected egg weight (W35) was 62 g. This aligns with the established understanding that heat stress reduces egg weight in poultry. Exposure to high ambient temperatures compromises thermoregulation in birds, negatively affecting their health and welfare. This stress can lead to decreased egg production and a reduction in the egg size and weight. Furthermore, heat stress can disrupt avian metabolism and affect feed intake, further contributing to decreased egg weight. In summary, extreme heat conditions significantly impact poultry egg production and quality [33-35]. The quality of the eggshell and the internal quality of the egg are of great importance to the egg industry. The quality of the eggshell can be assessed in terms of several parameters: egg size, the specific gravity of the egg, eggshell color, shell breaking strength, shell deformation, shell weight, shell percentage, shell thickness, and shell structure. These findings indicate that the quality of the eggshell can be positively affected by optimal dietary levels and types of manganese, as well as by the addition of prebiotics, probiotics, organic acids, and herbal extracts. Based on the results obtained, it is possible to assert that the antioxidants contained in the microalgae incorporated into the feed do not influence the eggshell quality, which is also an important concern for consumers, as strong resistance to breaking and lack of shell defects are essential for protection against the penetration of pathogenic bacteria into eggs [36]. The eggshell resistance variable (week 35) did not present significant differences between the treatments ($p > 0.05$), being their averages by treatments T4 5.2 ± 0.49; T3 5.05 ± 0.49; T2 5.0 ± 0.49; T5 4.96 ± 0.49 and T1 4.47 ± 0.49 Kgf (Figure 6B).

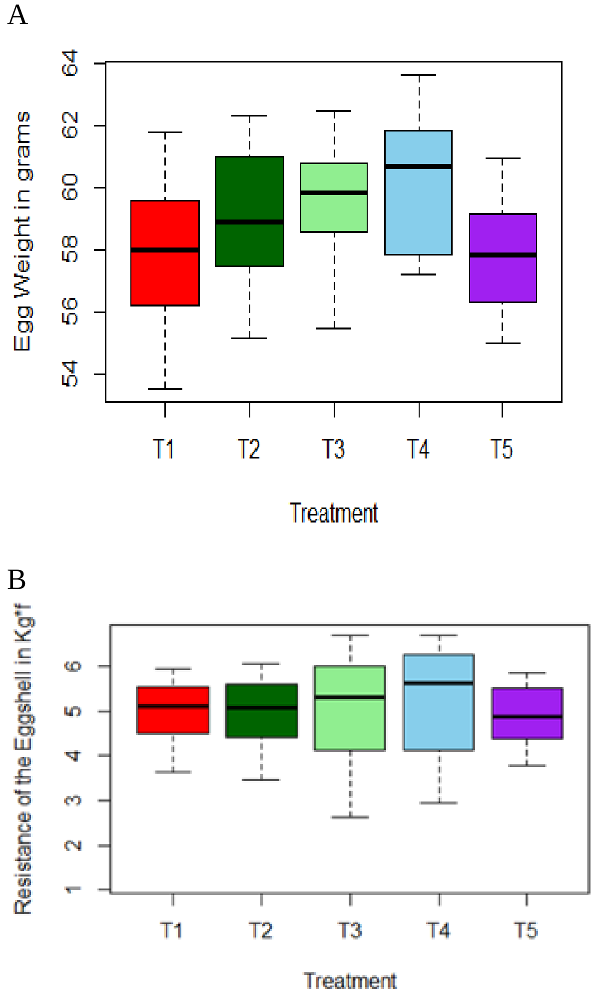


Figure 6. A) Egg weight (g) in week 35 and B) Eggshell resistance (Kgf): Treatment 1 (control group T1, without microalgae), T2, T3 and T4

(*Spirulina* and *astaxanthin)* and T5 (only *Spirulina*) (developed by the authors)

Similarly, there were no significant differences in the eggshell thickness at week 36 between treatments (p > 0.05) (

Figure **7**). Thickness and pigmentation are directly or supposedly related to the strength of the eggshell [36]. In addition, other factors interfere with the mineralization and quality of the shell, including environmental and nutritional factors, as well as the state of health and well-being of commercial hens [37]. In this case, the birds were in a free state, a situation that favors maintaining the quality of the shell, despite the fact that they were subjected to thermal stress.

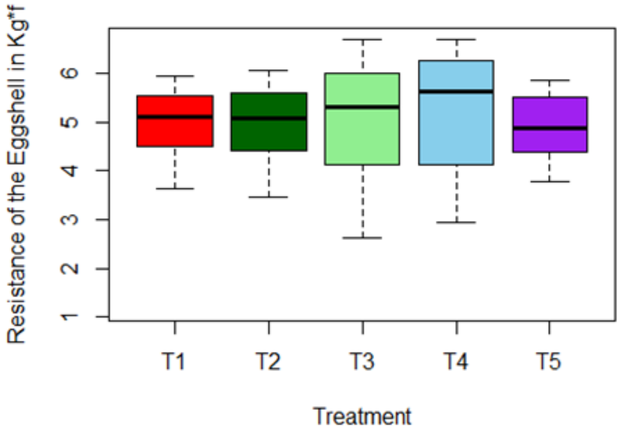
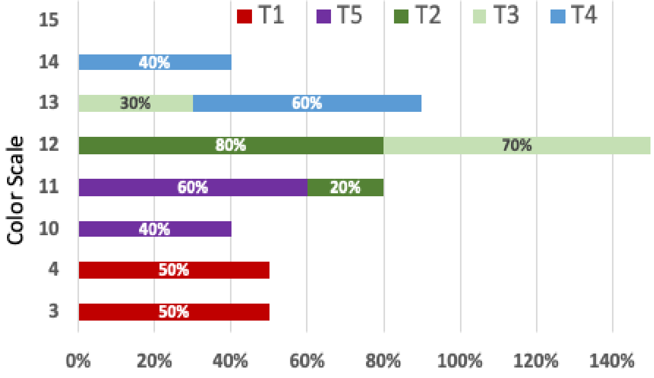


Figure 7. Eggshell thickness at week 35 between treatments (T1, T2, T3 y T4 and T5) (developed by the authors).

3.5. The Egg Yolk Color at Week 35

Estimations of the egg yolk color at week 35 (DSM Yolk Color Fan) showed that within the color scale, the T4 achieved the highest value being 40% of the buds examined on scale 14 and 60% on scale 13. This is followed by the T3 treatment of 70% on a scale of 12 and 30% on a scale of 13, for T2, their values are 80% on a scale of 12 and 20% on a scale of 11, and for T5 60% in 11 and 40% in scale 10 (

Figure **8**).



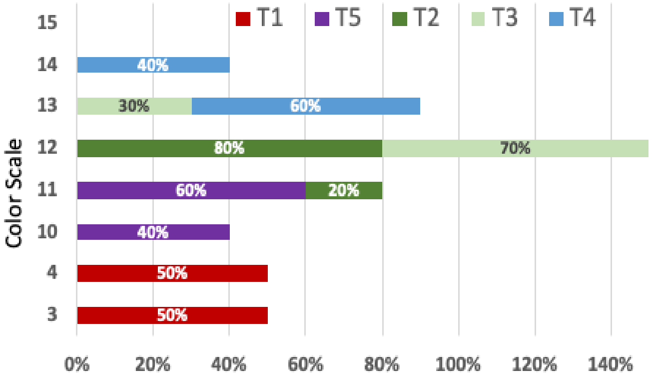


Figure 8. Yolk color was assessed at week 35 of production using the DSM Yolk Color Fan to compare the effects of the five treatments (T1, T2, T3, T4, and T5) (developed by the authors).

Finally, the T1 control group only achieved 50% in 4 and 50% on scale 3. For T5 treatment that only had Spirulina in the feed-in 1%, a significant improvement was found in egg yolk color in response to Spirulina supplementation [38]. In this study, T5 achieved a 2.75-fold increase in the color scale compared with the control group. On the other hand, T4, which includes both Spirulina and *Haematococcus pluvialis* in the feed, achieved a 3.5-fold increase in color compared with the control. As Spirulina is added and gradually the amounts of natural astaxanthin in the feed of the hens are increased, the color of the egg yolk increases reaching a maximum of 14, under conditions of thermal shock.

3.6. Carotenoid Content and Its Effects

Concerning the presence of carotenoids in the fresh yolk at week 35, the results show that there are significant differences between all treatments ($p < 0.05$). This pattern mirrors the yolk color assessment. There is a high correlation between the carotenoid content and the yolk color [39] (Figure 9). The T4 treatment achieved 270% ± 37% more carotenoids compared to the T1 control group, followed by T3 with a 198%± 40%, T2 achieved 174% ± 28% and the T5 group that has the addition in the feed 1% of Spirulina achieved158% ± 22% concerning the control. By gradually adding Spirulina and natural astaxanthin of *Haematococcus pluvialis* to the treatments T2, T3, and T4, the amounts of carotenoids in the egg yolk increased, reaching a maximum in the treatment T4. Other studies showed that the addition of microalgae in low percentages increases the carotenoid content in the egg yolk [40, 41].

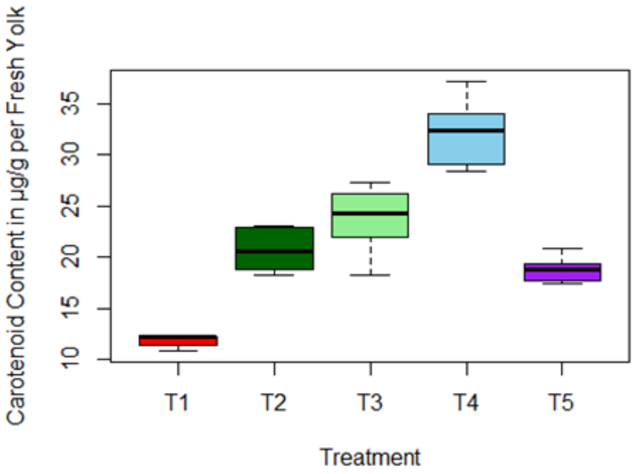


Fig. 9. Carotenoids in fresh yolk at week 36 between treatments (T1, T2, T3 y T4 and T5) (developed by the authors).

Following the weight loss observed in treatment T1 during week 36, treatments T1, T2, T3, and T5 were fed with the T4 diet. In this case, significant differences were observed between the weights of the treatments. However, by week 43, the weights of the treatments stabilized, and no significant differences were found, with the weights per treatment as follows: T1 2.14 ± 0.49; T2 2.16 ± 0.49; T3 2.14 ± 0.49; T4 2.15 ± 0.49 and T5 2.15 ± 0.49 Kg. It is interesting to note that treatments T1 and T5, by incorporating astaxanthin into their diets, were able to recover weight in response to thermal stress, especially considering that the transition into the winter months was underway (Fig. **10**).

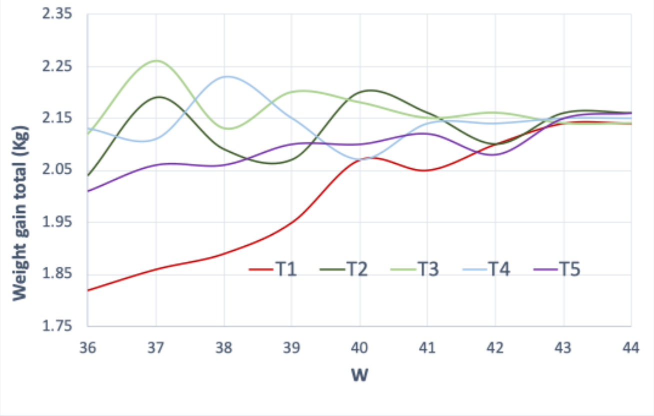


Fig. 10. Effect of the T4 diet on treatments T1, T2, T3, and T5 (The authors).

3.7. Effectiveness of the Microalgae for the Immune Response of Laying Hens

The results showed that the levels of antibodies against New Castle decreased when applying treatments T2, T3, T4, and T5 compared with treatment T1, which included the vaccine and feeding without spirulina and astaxanthin. This suggests that the effect of spirulina and astaxanthin in certain doses can interfere with the immune status of laying hens (Figure 11).

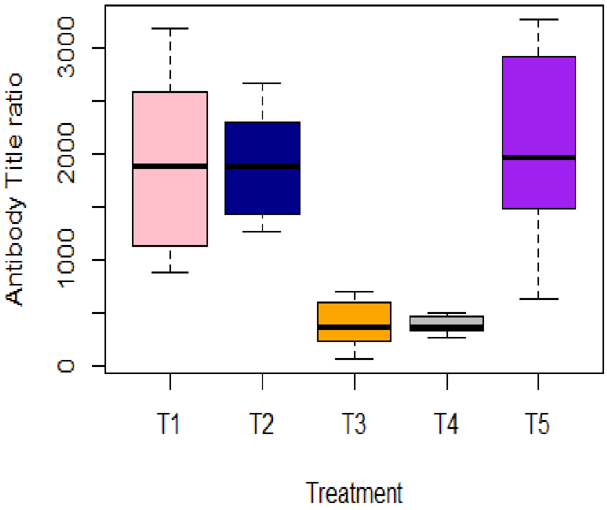


Figure 11. Effectiveness of the microalgae for the immune response of laying hens by applying the vaccine against the Newcastle Disease virus (developed by the authors)

It should be noted that the amount of spirulina for treatments T2, T3, T4, and T5 is the same, which indicates a similar inhibitory effect on immunity, as reported by another author [42], while the concentration of astaxanthin is only present in treatments T2, T3 and T4, at incremental doses of 20, 40 and 60 ppm respectively. These results show us an extra inhibitory effect of astaxanthin on the level of antibodies, especially in T3 and T4, where there is high dose astaxanthin supplementation. In the same way, as it has been supported by other authors, indicating that an appropriate amount of Ax must be evaluated [43, 44].

4. Conclusions

Supplementation with microalgae (*Spirulina* and *Haematococcus pluvialis*) proved to be an effective strategy in increasing egg production and body weight of laying hens, particularly under heat-stress conditions. Higher dietary concentrations of antioxidants benefited the birds' performance, such that they exceeded the performance of the treatment groups and the industrial reference values. Although, as expected, heat-stress affected egg weight, some average values did not significantly differ in terms of egg weight, shell strength, and shell thickness between the treatment groups. However, the yolk color, which is largely influenced by the carotenoid content, increased significantly with higher microalgae supplementation. The T4 treatment, which had higher inclusions of astaxanthin, resulted in the hens having the deepest yolk color and the highest carotenoid concentrations. Carotenoid-enriched eggs develop positive attributes that contribute to consumer health through their antioxidant characteristics. Subsequent weight loss in the control group required a diet change, and all groups were switched to the T4 diet. By week 43, the weights had stabilized, and astaxanthin supplementation appeared to mitigate the negative effects of thermal stress, suggesting a protective role of microalgae against heat-induced oxidative stress.

This work presents novel findings on the effects of supplementing laying hens' feed with two types of microalgae on egg quality and the hens' immune system. The supplementation enhances the antioxidants in the yolk, thereby improving the nutritional value of the eggs for human consumption. The innovation lies in the production of eggs with more than double the antioxidant content in the yolk compared to conventional eggs and in the ability to mitigate heat stress in the laying hens without compromising their productivity. Therefore, future research should focus on determining the optimal dosage of astaxanthin (Ax) to avoid immune suppression while potentially leveraging other beneficial effects. Careful consideration of the interaction between spirulina and astaxanthin is also warranted.

Declarations

Author Contributions

Conceptualization, J.P.D.; methodology, J.P.D.; validation, J.P.D., and C.I.; formal analysis, J.P.D.; statistical analysis, C.I.; investigation, including the immune system verification experiments and analysis J.P.D., F.G.A., K.F., F.D., and N.L.; data curation, J.P.D..; writing—original draft preparation, J.P.D., and C.I.; writing—review and editing, C.I.; visualization, K.F., and F.D.; supervision, J.P.D., and F.G.A.; funding acquisition, J.P.D.; project administration, J.P.D. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Institutional Review Board Statement

Rigorous ethical guidelines were adhered to throughout the study to ensure participant privacy and data confidentiality, in compliance with institutional and national research standards.

Informed Consent Statement

Participation in the study was voluntary and informed consent was obtained from all participants prior to their involvement.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this manuscript. In addition, ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies, have been completely observed by the authors.

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