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Sustainable microalgal feed formulations for functional and omega-3-enriched eggs in poultry production☆

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ABSTRACT

This study evaluated the dietary supplementation of *Hy-Line Brown*® laying hens with a microalgal blend composed of *Microchloropsis gaditana* and *Muriellopsis* sp. strain MCH-35 at inclusion levels of 1% and 3% (microalgal mix) and 10% (*M. gaditana*), aiming to improve the nutritional and functional quality of eggs. The formulated pellets were analyzed for proximate composition, fatty acid profile, carotenoid content, and antioxidant capacity, while the feeding trials were conducted over eight weeks. Microalgal inclusion enhanced the nutritional profile of the feed by increasing protein, lipid, and mineral contents while reducing crude fiber. *M. gaditana* contributed high levels of eicosapentaenoic acid (EPA), whereas *Muriellopsis* sp. exhibited higher lutein and total carotenoid contents, significantly increasing antioxidant capacity. In egg yolks, supplementation produced a progressive, dose-dependent increase in pigmentation, with the most intense coloration observed in the 3% and 10% treatments. Additionally, a significant increase in docosahexaenoic acid (DHA) and a reduction in linoleic acid (n-6) were recorded, improving the n-6/n-3 ratio from 24:1 (control) to 7:1 (3%) and 6:1 (10% *M. gaditana*). Total carotenoid and lutein contents in yolks increased by 2.3- and 2.7-fold, respectively, without affecting egg physical parameters. Biochemical profiles of the hens confirmed the absence of hepatic or metabolic stress, validating the safety of supplementation. Microalgae-enriched pellets offer an innovative and sustainable feed alternative that enables the production of functional eggs enriched with omega-3 fatty acids and carotenoids, enhancing their nutritional quality while adding both economic and environmental value to the poultry sector.

1. Introduction

One of the most serious health problems currently facing the global population is related to non-transmissible or metabolic diseases, which largely originate from poor eating habits and unbalanced diets consumed worldwide [1]. As a result, the prevalence of conditions such as obesity, diabetes, and cancer, among other pro-inflammatory and degenerative diseases, has increased exponentially [2–4]. This situation has led to a new wave of social awareness regarding the foods offered and consumed daily, thus driving a growing demand for healthy products of high or improved nutritional quality [1,5].

Among the foods that stand out for their high nutritional value, popularity, and accessibility, the egg stands out as a food characterized by its high content of proteins, lipids, vitamins, minerals, and antioxidants, which play a fundamental role in physiological well-being [6,7]. Furthermore, it has been shown that the nutritional composition of eggs can be modulated by the diet provided to laying hens [5,8], presenting a promising opportunity to produce a functional food that could help reduce malnutrition rates and improve quality of life on a global scale [9,10]. In this context, some of the key compounds of interest are lutein and omega-3 fatty acids, which are already present in eggs and are widely recognized for their health benefits. On one hand, lutein, which

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belongs to the carotenoid group, has strong antioxidant activity and is particularly known for its ability to prevent macular degeneration, along with its anti-inflammatory effects and other health benefits [6,11,12].

On the other hand, omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are highly valued for their cardiovascular, brain, and joint health benefits [13–15]. It is important to note that these compounds are not synthesized by the human body and must therefore be obtained through diet to gain their benefits [16].

In conventional feeding practices aimed at increasing the lutein and omega-3 content in eggs, pellets based on marigold or corn flour, seeds such as flaxseed or chia, and fish meal and/or oils are commonly used [17–19]. However, both fatty acids and lutein are quite labile when they come from these biomasses, so the absorption capacity of both compounds by hens can be reduced and, consequently, affect the production of fortified eggs [20–22]. Furthermore, it should be considered that the massive use of land for the cultivation of these raw materials represents a limitation from an environmental perspective [23], as well as the use of ingredients from fish derivatives, since it has been shown that they alter certain sensory characteristics of the eggs obtained (fishy taste and smell), which decreases their commercial acceptance [24,25]. Due to the above, new alternatives are being explored for effective supplementation of laying hens and thus obtain premium quality eggs (high in bioactive compounds) through approaches that are sustainable and economically viable for implementation in industrial production [23,26].

Recent reviews have highlighted that dietary inclusion of microalgae can modify the physical, chemical, and nutritional characteristics of poultry products, including eggs, with most benefits attributable to their unique biochemical composition, although some underlying mechanisms remain to be fully elucidated [27].

Among these alternatives, microalgae stand out as ideal candidates due to their high efficiency in biomass production and their ability to synthesize a wide array of high-value compounds, such as lipids, pigments, proteins, and minerals [28–30]. In particular, supplementation with carotenoid-rich species such as *Dunaliella salina* has been shown to improve poultry performance, modulate lipid metabolism, and enhance immune responses, supporting the use of microalgae as functional feed ingredients in animal production systems [31].

This study focuses on two microalgal species of biotechnological relevance: *M. gaditana*, recognized for its capacity to synthesize omega-3 polyunsaturated fatty acids [14,32], and the chlorophyte microalga *Muriellopsis* sp. strain MCH-35, distinguished by its exceptional pigment productivity, particularly lutein [33,34]. Remarkably, *Muriellopsis* sp. can accumulate lutein at levels five- to tenfold higher than those typically achieved by the conventionally used marigold (*Tagetes erecta*) [35]. Furthermore, as a fat-soluble carotenoid, lutein tends to accumulate more in the presence of lipids, which makes its combination with microalgal oils and the fatty matrix of the egg an environment with the optimal conditions to effectively supplement polyunsaturated fatty acids together with lutein [8,9].

Given the increasing interest in natural and sustainable sources of omega-3 fatty acids and carotenoids for functional food applications, microalgae have emerged as promising ingredients for improving the nutritional and bioactive profiles of animal-derived products. Therefore, this study aimed to evaluate the effects of dietary supplementation with *M. gaditana* and *Muriellopsis* sp. on the fatty acid composition and lutein content of eggs produced by laying hens. The main goal was to enhance the nutritional and functional quality of eggs through the incorporation of microalgae-derived bioactives.

2. Materials and methods

2.1. Cultivation conditions and scaling of the microalgae M. gaditana and Muriellopsis sp. strain MCH-35

The microalgae *Microchloropsis gaditana* (previously named *Nannochloropsis gaditana,* Lubián CCMP 527) [14,36] and *Muriellopsis* sp. [37], were cultivated both in laboratory and outdoor environments at the facilities of the Applied Microbiology Unit of the Centro de Bioinnovación (CBIA) at the Universidad de Antofagasta, located in Antofagasta, Chile (23° 42′ 19.0584″ S, 70° 25′ 20.9172″ W). In the laboratory, the culture was initiated in 2-liter glass flasks and, 15 days later, transferred to 20-liter polycarbonate bottles containing seawater filtered to 1 μm. The seawater was chlorinated and subsequently neutralized with sodium thiosulfate in a 3:1 ratio. The cultures were inoculated with 12% of the total volume. Laboratory conditions included a controlled temperature of 20 °C, continuous aeration, constant light exposure (24:7), and a culture duration of 15 days. The UMA-5 culture medium (modified f/2 medium enriched with $NaNO_3$ and NaH_2PO_3) ($NaNO_3$ 0.4 g L^{-1}, NaH_2PO_4 0.034 g L^{-1}, $NaHCO_3$ 0.168 g L^{-1}, Zn 0.080 μmol L^{-1}, Mn 0.9 μmol L^{-1}, Mo 0.03 μmol L^{-1}, Co 0.05 μmol L^{-1}, Fe 11.7 μmol L^{-1}) [36], was used throughout all culture stages. Subsequently, the cultivation was scaled up to large-scale flat-panel systems located outdoors at the Pilot Plant, with a capacity of 400 L. These systems were inoculated with 20% of the inoculum produced under the aforementioned laboratory conditions, and aeration was maintained by a constant airflow supplied by a blower, with a flow rate of 100 to 130 L min^{-1}. Finally, for the scaling up of the culture to 3.8 m^3 raceway systems, the inoculum from the 400 L flat-panel systems was used, transferring 20% of this inoculum to each of the new outdoor cultivation systems, which were operated under the cold desert climate conditions characteristic of the region (BWh) according to the Köppen-Geiger classification [38].

2.2. Analysis of microalgal biomass

2.2.1. Carotenoids extraction

The extraction of pigments was performed according to [39]. Briefly, 10 mg of alumina were mixed in a 1:1 ratio with the microalgal biomass, and 1 mm glass beads were added to the tube together with 1 mL of a three-component mixture (77% ethanol, 17% hexane, 6% H_2O, and 0.1% KOH). The tubes were processed in a bead beater for 2 min with 30 s pauses. The mixture was then centrifuged at 4500 rpm for 3 min, and the organic fraction was collected, filtered through 0.22 μm nylon membranes, and stored in amber vials until chromatographic analysis.

2.2.2. Lutein quantification utilizing high-performance liquid chromatography (HPLC)

The analysis of lutein in organic extracts was performed utilizing HPLC (LC-4000, Jasco, Japan), equipped with a quaternary pump (PU-2089s Plus, Jasco, Japan), diode array detector (MD-4010, Jasco, Japan), automatic injection system (AS-2055 Plus, Jasco, Japan), and ChromNAV Control Center V.2 software (Jasco, Japan). A reversed-phase column (LiChrosphere RP-18 HPLC column, 5 μm particle size, dimensions 150 mm × 4.6 mm) was employed. The gradient program was implemented as outlined by [40], and the injected volume of each sample was 20 μL. The mobile phase consisted of a mixture of two solutions: solution A was composed of water and methanol in a 2:8 volume/volume ratio, while solution B comprised acetone and methanol in a 1:1 volume/volume ratio. Carotenoids were eluted at a flow rate of 1 mL min^{-1}, and lutein was quantified through integration at a wavelength of 450 nm. Identification of lutein was accomplished by comparing retention times, calibration curves, and UV-VIS absorption spectra with a lutein standard (Sigma-Aldrich, St. Louis, MO, USA).

2.2.3. Determination of total proteins in microalgal biomass

Protein concentration in microalgal biomass was determined using a modified Lowry method [41]. The following reagents were prepared: (i) 5% sodium carbonate (Na_2CO_3) solution (R1); (ii) 0.5% copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) in 1% sodium potassium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$) (R2); (iii) freshly prepared Cu–tartrate reagent (R3) obtained by mixing R1 and R2 immediately before use; and (iv) Folin–Ciocalteu reagent diluted 1:1 (*v*/v) with distilled water (R4).

For extraction, 15 mg of dry biomass were suspended in 2.0 mL of 1 N NaOH and incubated in a thermostatic water bath at 95–100 °C for 1 h. The samples were then centrifuged at 3000 rpm for 5 min, and the supernatants were collected for subsequent analysis.

Aliquots of 0.1 mL of each supernatant were transferred (in triplicate) to clean tubes, and the volume was adjusted to 0.3 mL with 1 N NaOH. Then, 0.4 mL of distilled water were added. A colorimetric blank was prepared with 0.4 mL of 1 N NaOH and 0.4 mL of distilled water, while a positive control contained 0.1 mL of bovine serum albumin (BSA) stock solution (1 mg mL^{-1}), 0.3 mL of distilled water, and 0.4 mL of 1 N NaOH.

For quantification, 2.0 mL of the freshly prepared R3 reagent were added to all tubes, which were then vortexed and allowed to react for 10 min. Subsequently, 0.4 mL of the diluted Folin–Ciocalteu reagent (R4) was added and mixed immediately. The mixtures were incubated for 30 min at room temperature in darkness, and absorbance was measured at 750 nm using a 1 cm quartz cuvette.

A standard calibration curve was constructed using serial dilutions of BSA according to the manufacturer's instructions.

2.2.4. Determination of total lipids and fatty acids in microalgal biomass

Lipid determination was conducted using the Kochert method [42], subsequently followed by the isolation of the organic phase via evaporation and concentration of the extract. The fatty acid profile was obtained through direct transesterification and gas chromatography (GC), as elucidated by Rodríguez-Ruiz et al. [43].

2.3. Preparation and analysis of feed

2.3.1. Supplementation of conventional feed for laying hens with microalgae mix rich in lutein and omega 3

To supplement the diet of laying hens with microalgae, a pelletized feed was prepared by mixing conventional feed with a microalgal blend rich in lutein and omega-3, derived from the *Muriellopsis* sp. and *M. gaditana*, respectively. Pellet production was carried out using a pelletizer with a capacity of 250–350 kg h^{-1} (model TMR-Z300BM, Indusus SpA, Chile), equipped with a stationary die of 4 mm. Four pellet prototypes were formulated according to the percentage of microalgal supplementation in the feed:

(i) Feed +0% microalgae (control)
(ii) Feed +1% *Muriellopsis* sp. + *M. gaditana*
(iii) Feed +3% *Muriellopsis* sp. + *M. gaditana*
(iv) Feed +10% *M. gaditana*.

The pellets had a diameter of 1 cm and a moisture content of 2.5% (Fig. 1).

2.3.2. Program for feeding laying hens with microalgae-supplemented pellets

Starting from the onset of laying (week 18), 90 Hy-Line Brown® hens from a batch of 2500 birds born on March 28, 2024, at the El Monte hatchery, located in the metropolitan region, exclusive distributor of Hy-Line® in Chile, were distributed into 4 treatment groups according to the percentage of microalgal supplementation in the feed. The feed was provided daily according to the amounts recommended by Hy-Line, and water was supplied ad libitum. Once a month, Promotor L® was added to the water for 5 consecutive days to correct possible deficiencies in amino acids and vitamins.

2.3.3. Analysis of microalgae-supplemented pellets for feeding laying hens

After preparation, the pellets were vacuum-sealed and transported to the Institute of Nutrition and Food Technology (INTA) at the University of Chile for comprehensive analyses, including proximate composition, fatty acid profile, total carotenoid content, and antioxidant activity.

2.4. Analysis of eggs

2.4.1. Physical measurements of egg samples

Several egg quality parameters were evaluated, including total egg weight, albumen weight, yolk weight, shell thickness, and yolk coloration. Seven yolks were collected for analysis. All weights were recorded using a digital balance, while shell thickness was measured with a digital force gauge micrometer (SHSIWI, China). The egg yolk coloration was measured using the dsm-firmenich Yolk Color Fan (coloration index range 1–15; DSM, Switzerland).

2.4.2. Analysis of total lipid composition in egg yolk

For the analysis of yolk lipids, the solvent extraction method (OBD) described by Schreiner [44], was employed with modifications. Six yolks were collected and randomly pooled in pairs to obtain three replicates. The samples were frozen at −80 °C and subsequently lyophilized using a freeze-dryer (INOFD-18P, INNOVA). From each replicate, 0.5 g of dried yolk were accurately weighed and homogenized in 1.6 mL of distilled water for 90 s. A mixture of 4 mL chloroform and 4 mL methanol was then added, followed by an additional 90 s of stirring. Subsequently, 2 mL of distilled water were introduced and briefly mixed for 5 s. The tubes were centrifuged at 3000 rpm for 10 min, and the lower

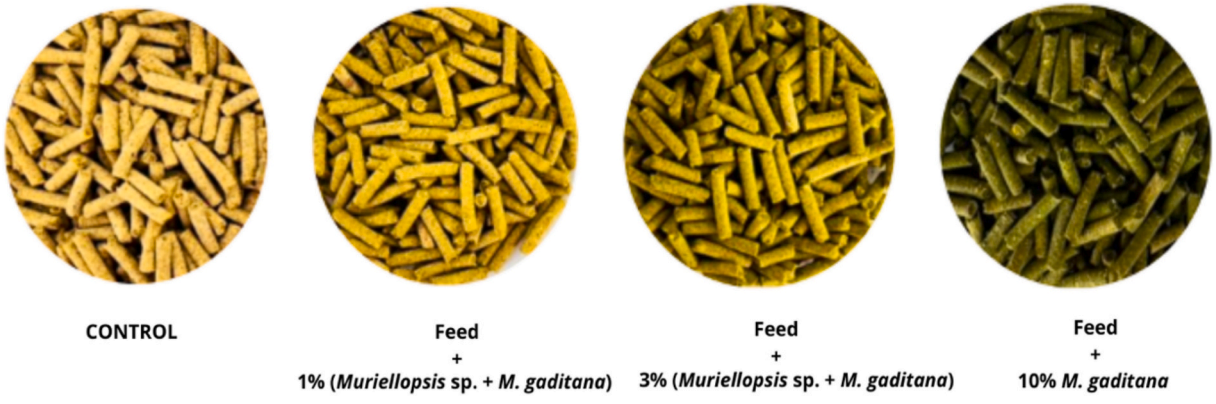


Fig. 1. Pellet prototypes microalgae-enriched for laying hen feed.

lipid-containing phase was collected in pre-weighed clean tubes. This extraction procedure was repeated three times, each time with the addition of 4 mL chloroform, centrifugation, and recovery of the lipid phase.

2.4.3. Fatty acids and total carotenoids in egg yolk

For sample preparation, 30 egg yolks were collected from each treatment and homogenized using a digital Ultraturrax homogenizer (model RW 20, IKA, Germany) to obtain a representative sample. The homogenates were then dehydrated using a freeze-dryer (INOFD-18P, INNOVA), vacuum-sealed, and subsequently sent to the Institute of Nutrition and Food Technology, University of Chile (INTA), at three different time points: T0 (week 0), T4 (week 4), and T8 (week 8), for fatty acid and total carotenoid analyses.

2.4.4. Lutein quantification in egg yolk

Lutein quantification was performed following a saponification protocol adapted from Wang et al. [45]. Briefly, 50 mg of dehydrated egg yolk, obtained from the representative sample, were weighed, and mixed with 10 mL of an ethanolic potassium hydroxide solution (45% KOH in 95% ethanol). Samples were incubated for 1 h at 60 °C with intermittent vortex agitation. After incubation, the solvent was evaporated to concentrate the extracts, which were then resuspended in 2 mL of a ternary solution as described by Cruz-Balladares et al. [33]. The resulting mixtures were vortex-homogenized, filtered through 0.22 μm nylon membranes, and stored in amber vials until chromatographic analysis.

2.4.5. Quantification of lutein and antioxidant activity in egg yolk

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as described by Diaz-MacAdoo et al. [34]. Briefly, 100 μL of the previously obtained lutein extracts were mixed with 150 μL of 0.2 mM DPPH solution. The samples were incubated for 30 min in the dark and then measured at 517 nm using a spectrophotometer. Results were expressed as percentage inhibition (%).

2.4.6. Physiological parameters of laying hens

For the biochemical profile analysis of the laying hens, five birds were randomly selected from each experimental group. Blood collection was performed by a collaborating veterinarian via the brachial vein, following the protocol recommended by the College of Veterinary Medicine at Western University of Health Sciences ("Poultry Blood Collection"). A maximum volume equivalent to 1% of body weight was extracted using a 25G needle after disinfecting the site with 2% chlorhexidine. The samples were transferred to tubes for serum biochemical testing. To monitor these parameters, blood was collected at two time points during the experimental phase:

i) T4 = Week 4 (prior to supplementation)
ii) T8 = Week 8 (last day of supplementation)

Samples were submitted to an external laboratory for the analysis of glucose, blood urea nitrogen, creatinine, total protein, albumin, calcium, phosphorus, total bilirubin, alkaline phosphatase, GOT (AST), GTP (ALT) and others. These measurements provided an overview of the metabolic and physiological status of the hens during the feeding trial.

2.5. Statistical analysis

All experiments were conducted in triplicate to ensure reproducibility, except for the physical quality analyses of the eggs, which were carried out with seven replicates. Data are presented as mean ± standard deviation (SD). Total carotenoids and ORAC content in the pellet were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, with statistical significance set at $p \leq 0.05$. Total carotenoids and lutein content in the yolk were analyzed using two-way ANOVA with Tukey's post hoc test, with statistical significance also set at $p \leq 0.05$. All analyses were performed using GraphPad Prism version 9.2.0.

3. Results and discussion

3.1. Microalgal biomass analysis

The comparison between *M. gaditana* and *Muriellopsis* sp. revealed significant differences in their biochemical composition (Table 1). *M. gaditana* exhibited a markedly higher content of proteins (272.78 mg/g) and total lipids (289.26 mg/g) in comparison with *Muriellopsis* sp., consistent with the ability of this microalgal species to accumulate elevated levels of these biocompounds [46]. Regarding the lipid profile, *M. gaditana* was characterized by the presence of eicosapentaenoic acid (EPA, 0.99 mg/g), whereas *Muriellopsis* sp. was distinguished by its enrichment in n-6 fatty acids, particularly linoleic acid (LA, 2.01 mg/g) and γ-linolenic acid (GLA, 8.79 mg/g), the latter being a key bioactive precursor of arachidonic acid [47]. These fatty acids are also of important health value since, like n-3 fatty acids, their deficiency has been associated with various diseases, such as dermatitis, arthritis, and diabetes, among others [48]. Therefore, supplementing hens with n-6 fatty acids will allow this family of fatty acids to be incorporated into eggs [49,50].

In contrast, regarding antioxidant compounds, *Muriellopsis* sp. displayed a higher total carotenoid content (2.24 mg/g vs 1.73 mg/g) and, notably, more than twice the lutein concentration detected in *M. gaditana* (6.19 vs. 2.76 mg/g). This can be attributed to the fact that this microalga accumulates free lutein as its main carotenoid [51]. The contribution of carotenoids by *Muriellopsis* sp. would also have positive effects on hens, since it has been described that carotenoids modulate certain molecular processes that allow an increase in egg production and their size [8,52].

In addition, the fact that these pigments come from microalgal sources rich in lipids, promotes better absorption by the metabolism of hens due to the liposulibility of carotenoids.

3.2. Formulation of microalgae-supplemented poultry feed and proximate composition analysis

The microalgae-supplemented pellets were adjusted to a diameter of 1 cm and a moisture content of 2.4%, in order to ensure feed acceptance by the hens and to achieve successful palleting in terms of product consistency and durability, while also preventing potential clogging of the pelletizing machine.

The incorporation of microalgae into poultry feed was associated with variations in proximate composition and fiber content, as shown in Table 2. Moisture content was lower in all experimental treatments compared to the control (18.2%). Reduced moisture levels may contribute to better shelf stability by limiting water availability, thereby helping preserve feed quality over time.

Table 1
Comparative composition of lipids, fatty acids, and carotenoids in *M. gaditana* and *Muriellopsis* sp.

(mg/g)		*M. gaditana*	*Muriellopsis* sp.
Protein		272.78	58.08
Total lipid		289.26	123.8
Fatty acid	C18:2n6 Linoleic acid	0.27	2.01
	C18:3n6 Gamma-linolenic acid	–	8.79
	C22:6n3 Eicosapentaenoic acid (EPA)	0.99	–
Total Carotenoids		1.73	2.24
Lutein		2.76	6.19

Table 2
Proximal analysis and fiber in different microalgae-supplemented pellets.

Results (per 100 g feed)	Control	Feed + 1% microalgal mix	Feed + 3% microalgal mix	Feed + 10% *M. gaditana*
Moisture (g)	18.2	14.9	15.2	15.3
Ash (g)	12.5	13.2	13.7	14.9
Protein (g)	12.8	13.3	13.7	13.7
Total fat (g)	3.8	4.2	4.3	4.4
Crude fiber (g)	3.0	2.3	2.7	2.4
Available Carbohydrates (g)	49.7	52.1	50.8	49.3
Energy (kcal)	248	299	295	292

Ash content showed a gradual increase with higher levels of microalgal inclusion, from 12.5% in the control to 14.9% in the pellets enriched with 10% *M. gaditana*, suggesting an increase in mineral content derived from the algal biomass. The mineral composition of microalgae is a critical aspect that enhances their value as a potential component in poultry feed, thanks to their rich and varied nutritional profile. Overall, the diverse range of mineral content in these species highlights their potential as versatile and abundant sources of essential nutrients for poultry, underscoring the possibility of their use in enhancing poultry diets [53].

Protein content was slightly higher in all supplemented pellets (13.3–13.7%) compared to the control (12.8%). Total fat content exhibited a similar trend, increasing from 3.8% in the control to 4.4% in the feed supplemented with 10% *M. gaditana*. Available carbohydrate content ranged from 49.3 to 52.1%, with the highest value recorded in the 1% microalgal mix group and the lowest in the diet supplemented with 10% *M. gaditana*. These results can be attributed to the fact that, in general, proteins, lipids, and carbohydrates constitute the main biochemical components of microalgae [54].

Energy values were consistently higher in the supplemented pellets (292–299 kcal/100 g) relative to the control (248 kcal/100 g), reflecting the contribution of added proteins, lipids, and carbohydrates from microalgal biomass.

When comparing the formulations, crude fiber content was consistently lower in the microalgae-supplemented diets than in the control, as conventional poultry feed is naturally richer in fiber (approximately 6–8%). Since part of this feed was replaced by microalgal biomass, which contains lower fiber levels, the overall fiber content decreased. This reduction in fiber may influence digestibility and, consequently, the efficiency of nutrient utilization by the laying hens.

Overall, these compositional changes indicate that the inclusion of microalgae, particularly in diets supplemented with 3% of the microalgal mix and 10% *M. gaditana*, can improve the nutritional and energetic profile of the feed pellets, thereby offering potential benefits for laying hen diets in terms of both quality and value. These findings are consistent with previous studies showing that the replacement of soybean meal (SBM) with *Spirulina platensis* (SP) in layer diets improved productive performance and egg quality, with optimal effects at 9% SP inclusion. Furthermore, it was suggested that 1% SP can replace 4.7% SBM as a promising nutritional strategy to optimize laying hens performance [55].

3.2.1. Fatty acids composition in supplemented feed

The fatty acid composition of the different formulated pellets was evaluated to determine the effect of microalgal supplementation (Fig. 2 and Table 3). In general, the inclusion of microalgal biomass led to a progressive increase in the total fatty acid content of the pellets, from 3656.7 mg/100 g feed in the control to 4249.4 mg/100 g feed in the 10% treatment (+16.2%), without drastically modifying the relative distribution among lipid classes (Fig. 2). Polyunsaturated fatty acids (PUFA) represented the major fraction in all treatments (47.4–48.6%), followed by monounsaturated (MUFA) and saturated fatty acids (SFA). A slight decrease in MUFA (31.65% in the control vs. 29.96% in the 10% treatment) and a modest increase in SFA (20.37% in the control to 21.47% in the 10% treatment) were observed with higher supplementation levels, indicating that microalgae addition maintained the overall fatty acid balance of the pellets while enriching them in total lipids. Similar patterns have been reported in poultry diets supplemented with

Table 3
Polyunsaturated fatty acid profiles in different pellets supplemented.

Polyunsaturated fatty acids (mg/100 g feed)	Control	Feed + 1% microalgal mix	Feed + 3% microalgal mix	Feed + 10% *M. gaditana*
C18:2n6 Linoleic acid (LA)	1683.73	1838.67	1820.3	1856.25
C18:3n3 Alpha-linolenic acid (ALA)	70.49	79.23	85.47	90.95
C20:3n6 Dihomo-gamma-linolenic acid (DGLA)	0.0	0.0	0.0	2.24
C20:4n6 Arachidonic acid (ARA)	0.0	0.0	5.48	14.92
C20:5n3 Eicosapentaenoic acid (EPA)*	0.0	6.81	21.82	99.61
C22:6n3 Docosahexaenoic acid (DHA)	0.0	0.0	0.0	0.0
TOTAL	1754.22	1924.71	1933.07	2063.97

The asterisk higlights the presence of EPA in feeds supplemented with microalgae.

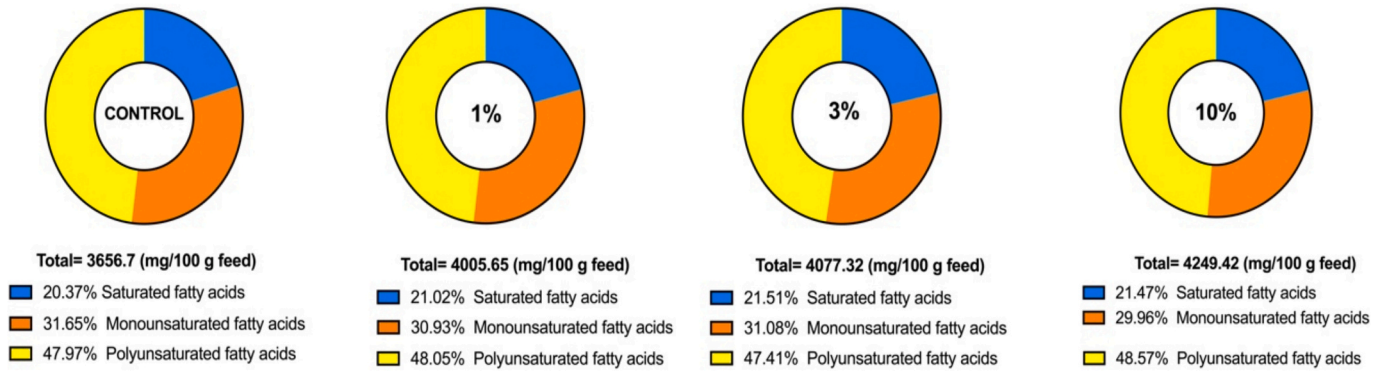


Fig. 2. Total lipid content (mg/100 g feed) in control feed and feeds supplemented with 1% and 3% microalgal mix (*Muriellopsis* sp. + *M. gaditana*) and 10% *M. gaditana*.

other microalgae such as *Chlorella sorokiniana*, *Spirulina platensis* or *Nannochloropsis gaditana*, which enhanced the total lipid and PUFA content of feed formulations without negative effects on nutritional performance [56,57]. The quantitative enrichment observed here is consistent with the intrinsic fatty acid composition of *M. gaditana* and *Muriellopsis* sp., both recognized as microalgae rich in unsaturated lipids [51,58,59].

The most relevant modification occurred in the qualitative composition of the PUFA fraction (Table 3). Eicosapentaenoic acid (EPA, C20:5 n-3), absent in control, appeared in the supplemented diets and increased dose-dependently, reaching 99.6 mg/100 g feed in the 10% *M. gaditana* treatment. Small amounts of dihomo-γ-linolenic acid (DGLA, C20:3 n-6; 2.24 mg/100 g) and eicosatetraenoic acid (ETA, C20:4 n-6; 5.48–14.92 mg/100 g) were also detected in the 3% and 10% groups, while docosahexaenoic acid (DHA, C22:6 n-3) remained undetected in all formulations. This pattern was expected, as *M. gaditana* is a well-known EPA-producing species with negligible DHA content [58–60]. In contrast, *Muriellopsis* sp. contributes mainly to C18:2 n-6 y C18:3 n-3 fatty acids, providing complementary PUFA profile content characteristic of the genus [51].

From a nutritional standpoint, the incorporation of EPA and minor amounts of DGLA and ARA is relevant since these long-chain PUFA are precursors of eicosanoids involved in inflammatory regulation [61]. Moreover, the improved n-6/n-3 balance in the supplemented feeds may contribute to the health value of animal-derived products.

Overall, these results demonstrate that microalgal supplementation increased the total lipid content of the formulated pellets while preserving a balanced fatty acid profile. Moreover, it qualitatively enhanced the polyunsaturated fatty acid (PUFA) pool through the incorporation of long-chain n-3 fatty acids, particularly eicosapentaenoic acid (EPA). These compositional improvements contribute to the nutritional upgrading of the feed, highlighting its potential functional role in enhancing n-3 fatty acid bioavailability for subsequent deposition in egg yolks during the feeding trial. The beneficial effects observed in our study are consistent with previous reports showing that microalgae such as *Dunaliella salina* can improve lipid profiles and immune function in poultry, likely due to their high content of carotenoids and polyunsaturated fatty acids [31].

3.2.2. Carotenoid content and antioxidant activity of different pellet formats

The total carotenoid content and antioxidant activity (ORAC) of the formulated pellets were evaluated to determine the effect of microalgal supplementation (Fig. 3). Both parameters increased significantly in all supplemented treatments compared with the control feed ($p < 0.05$). The inclusion of *M. gaditana* at 10% resulted in the highest total carotenoid concentration, followed by the 3% and 1% blends containing *Muriellopsis* sp. and *M. gaditana*. The antioxidant activity followed a similar trend, increasing proportionally with microalgal inclusion levels. This strong correlation between carotenoid content and ORAC values is expected, given that carotenoids act as efficient free-radical scavengers through their conjugated polyene structure, which determines their light-absorbing and antioxidant properties, enhancing the oxidative stability and functional quality of the feed [62,63].

The enrichment observed can be directly related to the pigment composition of the supplemented microalgae. *Muriellopsis* sp. has been described as a lutein-rich chlorophyte, with lutein representing more than 80% of its total carotenoids and concentrations reaching in range of 4.0 to 6.0 mg/g dry biomass under outdoor or high-irradiance conditions [34,51,62,64]. In contrast, *M. gaditana* exhibits a more complex pigment profile dominated by violaxanthin and β-carotene, together with vaucheriaxanthin esters that are characteristic of eustigmatophyte microalgae. These pigments, which form part of the xanthophyll cycle, play crucial photoprotective and antioxidant roles by dissipating excess excitation energy and maintaining redox balance under high irradiance [65–67]. The coexistence of these carotenoids provides *M. gaditana* with a broad antioxidant spectrum that complements the lutein-dominated profile of *Muriellopsis* sp., thereby explaining the higher total carotenoid content and enhanced ORAC values observed in the 10% supplemented treatment.

In summary, microalgal inclusion enhanced both the carotenoid and antioxidant profiles of the formulated feeds, confirming the suitability of *M. gaditana* and *Muriellopsis* sp. as complementary bioactive sources in functional feed formulations aimed at enriching animal products with natural antioxidants.

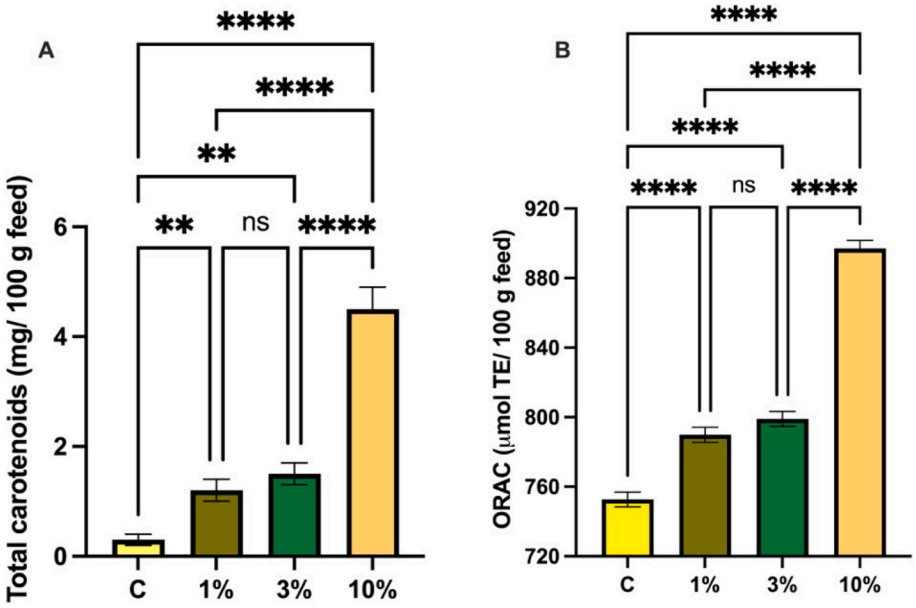


Fig. 3. Total carotenoid content and antioxidant capacity (ORAC) in formulated feeds supplemented with different percentages of microalgae. (A) Total carotenoid content (mg/100 g feed) and (B) antioxidant capacity expressed as ORAC (μmol TE/100 g feed) in control feed and feeds supplemented with 1% and 3% microalgal mix (Muriellopsis sp. + *M. gaditana*) or 10% *M. gaditana*. *Significantly different ($p < 0.05$). Values are based on the mean ± S.D. $n = 3$.

3.3. Yolk coloration analysis and physical parameters of eggs obtained from microalgae-supplemented feeding

Several egg quality parameters were evaluated, including yolk coloration (Fig. 4) and physical characteristics such as total egg weight, shell thickness, and albumen and yolk weights (Fig. 5).

Regarding yolk pigmentation, dietary supplementation with microalgae resulted in a proportional increase in yolk coloration across the 1%, 3%, and 10% treatments. This effect was more pronounced at higher inclusion levels (3% and 10%) and showed a progressive trend over time, becoming evident from week 4 onward, with significant increases in the yolk color index as measured by the Yolk Color Fan scale (Roche, DSM). By week 8, the treatments with feed +3% microalgal mix and feed +10% *M. gaditana* reached the highest values on the colorimetric scale, both significantly higher than the control and 1% (Fig. 4). The present findings are consistent with the study of Wu et al. [68], who reported that the partial substitution of soybean meal with *Nannochloropsis* sp. led to a significant increase in yolk pigmentation, shifting from yellow to reddish-orange after approximately seven days of supplementation. Similarly, another study investigating the dietary inclusion of the microalga *Isochrysis galbana* in laying hens demonstrated increased yolk darkness, reduced yellowness, and enhanced redness, particularly at supplementation levels ranging from 200 to 400 mg of microalgae per 100 g of feed, which could be attributed to the enrichment of fucoxanthin derivatives in the egg yolk [69]. These results confirm that the inclusion of microalgae in laying hen diets efficiently promotes the transfer of lutein and other carotenoids to the egg, mainly enhancing yolk pigmentation.

No adverse effects on the physical parameters of the eggs were observed following dietary microalgal supplementation. Average egg weight, as well as albumen and yolk weights, remained statistically similar among treatments throughout the feeding period. Likewise, shell thickness was unaffected, indicating that microalgae inclusion did not interfere with the process of shell mineralization. Overall, these findings confirm that neither egg size nor physical quality were compromised compared to the control group (Fig. 5). The lack of significant differences in shell thickness among treatments indicates that dietary microalgae did not impair shell mineralization. However, eggshell quality is a complex characteristic that cannot be fully assessed through thickness measurements alone. Parameters such as shell breaking strength, elastic deformation, porosity, specific gravity, and mineral composition are also important determinants of shell integrity and resistance to fracture. Consequently, although the present results suggest that microalgal supplementation did not negatively affect shell quality, future investigations should include these additional indicators to better characterize the potential effects of dietary microalgae on eggshell structure and mechanical performance.

The observed increases in yolk pigmentation and n-3 enrichment are consistent with previous evidence showing that microalgal supplementation efficiently promotes the transfer of carotenoids and long-chain omega-3 fatty acids (EPA and DHA) into the yolk, thereby improving the n-6/n-3 ratio [27]. These results also align with those reported by Wu et al. [68], who found that partial replacement of soybean meal with *Nannochloropsis* sp. did not significantly affect egg weight, yolk weight, albumen weight, or yolk-to-albumen ratio after a 28-day feeding trial, regardless of supplementation level.

Collectively, these findings reinforce that dietary inclusion of microalgae does not negatively impact the main physical traits of eggs. Interestingly, Panaite et al. [70] reported contrasting results, observing significant increases in egg weight after eight weeks of supplementation with 2% *Chlorella vulgaris* and 2% *Spirulina platensis* in Lohmann Brown laying hens at 38 weeks of age. Such discrepancies may be attributed to differences in genetic background, bird age at supplementation onset, or other experimental factors influencing productive performance.

3.4. Analysis of lipid and fatty acid composition in egg yolks from microalgae-supplemented diets

The total lipid content of egg yolks remained comparable across all treatments (approximately 61–65% of yolk dry weight), with no major differences observed in the levels of saturated and monounsaturated fatty acids. Similar results were reported by Wu et al. [68], where supplementation of hens with different concentrations of *Nannochloropsis* sp. did not alter the total fatty acid content or profile, at least up to 8% dietary inclusion.

In contrast, our study revealed a reduction in polyunsaturated fatty acids (PUFAs) in eggs from the 3% (feed +3% microalgal blend) and the 10% (feed +10% *M. gaditana*) treatments at week 8 (Table 4). This decrease may indicate a possible metabolic adaptation of the hens or altered absorption dynamics that affected PUFAs deposition in the yolk.

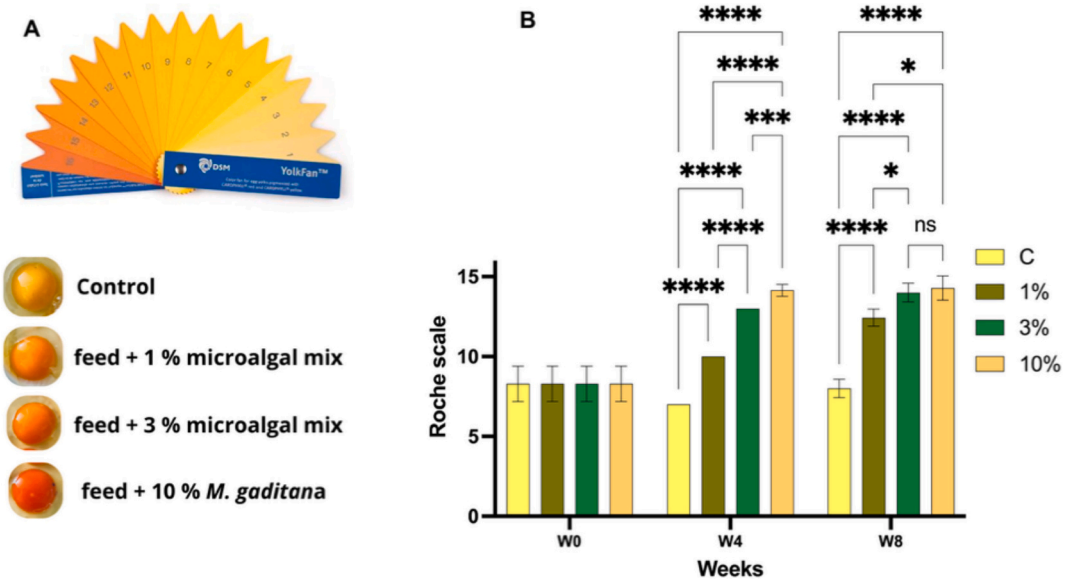


Fig. 4. Effect of yolk coloration obtained from feed with different microalgae supplementation proportions. (A) Yolk coloration obtained from hens fed the experimental diets (B) Quantitative evaluation of yolk color using the DSM Roche color scale at weeks 0, 4, and 8 of microalgal supplementation. *Significantly different ($p < 0.05$). Values are based on the mean ± S.D. $n = 7$.

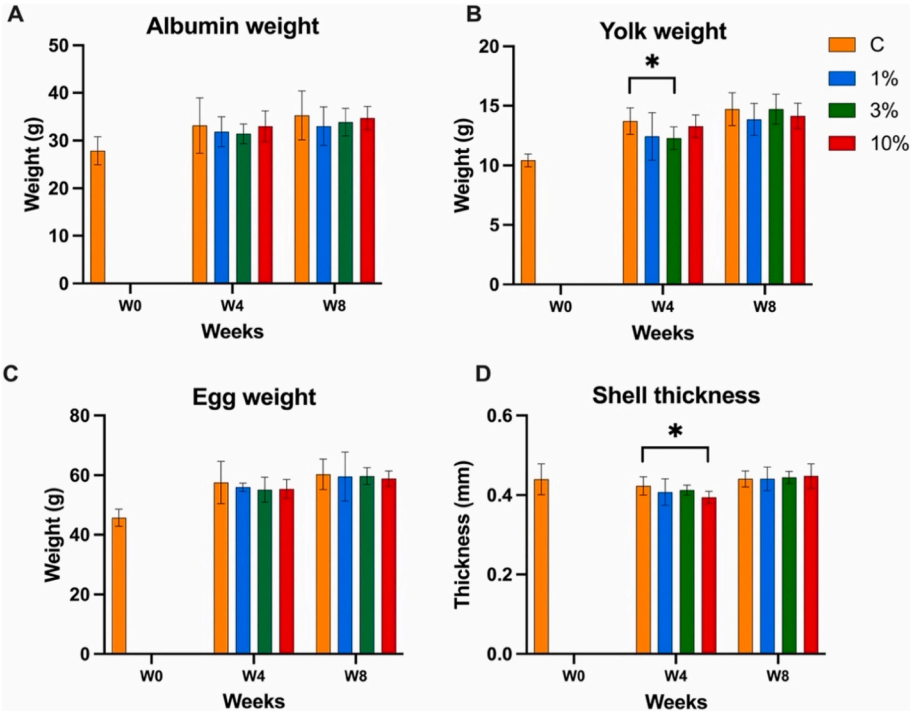


Fig. 5. Physical Parameters of the Egg. The weights of the whole egg (A), albumen (B), yolk (C), and shell thickness (D) were measured and illustrated in bar graphs comparing the feeding treatments across weeks 0, 4, and 8. *Significantly different ($p < 0.05$). Values are based on the mean ± S.D. $n = 7$.

Table 4
Analysis of total lipid and fatty acids content in lyophilized egg yolks.

Total lipids and Fatty acids (mg/100 g freeze-dried yolk)	Control		Feed + 1% microalgal mix		Feed + 3% microalgal mix		Feed + 10% *M. gaditana*	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Total Lipids	62,643.5	61,443.7	63,103.9	62,345.3	64,153.7	61,833.3	63,301.7	64,746.7
Saturated fatty acids	17,492.8	17,212.4	16,947.7	16,875.0	17,182.0	17,182.1	17,269.8	16,969.5
Monounsaturated fatty acids	21,032.5	20,782.2	20,643.2	20,873.2	21,507.9	21,247.4	20,789.1	20,821.0
Polyunsaturated fatty acids	8736.9	8956.3	8903.9	81,823.0	8627.3	7805.1	8838.9	7883.2

On the other hand, the analysis of the polyunsaturated fatty acid (PUFAs) profile in egg yolks collected at weeks 4 and 8 revealed a progressive decrease in omega-6 fatty acid content as a function of both time and the percentage of microalgae inclusion, with a more pronounced reduction observed in the 3% (feed +3% microalgal blend) and 10% (feed +10% *M. gaditana*) groups at week 8 (Table 5).

In parallel, an increase in omega-3 fatty acids was observed in the supplemented groups, particularly docosahexaenoic acid (C22:6n3, DHA). Compared with the control group (280.48 mg/100 g freeze-dried yolk) at week 8 of the diet, the 1% (feed +1% microalgal mix) showed a

Table 5
Analysis of polyunsaturated fatty acids content in lyophilized egg yolks.

Polyunsaturated fatty acids (mg/100 g freeze-dried yolk)	Control		1% (feed + microalgal mix)		3% (feed + microalgal mix)		10% *M. gaditana*	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
C18:2n6 Linoleic acid (LA)	7726.42	7333.31	7806.60	6667.84	7540.88	5963.78	7439.69	5809.7
C18:3n6 Gamma-Linolenic Acid (GLA)	0.0	51.77	0.0	44.95	0.0	29.79	0.00	37.09
C18:3n3 Alpha-linolenic acid (ALA)	126.14	175.76	163.74	156.50	146.58	166.71	162.61	150.32
C20:2n6 Eicosadienoic acid (EDA)	104.50	87.69	100.96	92.74	98.14	90.80	70.66	75.62
C20:3n6 Dihomo-gamma-linolenic acid (DGLA)	59.99	80.58	53.87	75.46	42.05	71.10	39.06	76.14
C20:4n6 Arachidonic acid (ARA)	595.88	914.94	612.28	807.05	595.59	697.16	641.07	726.66
C20:5n3 Eicosapentaenoic acid (EPA)*	0.0	0.0	0.0	0.0	0.0	24.53	0.0	51.04
C22:6n3 Docosahexaenoic acid (DHA)*	123.93	280.48	166.43	313.37	204.08	761.28	485.84	956.67

The asterisk highlights the presence of EPA and DHA in lyophilized egg yolks.

1.1-fold increase in DHA content (313.37 mg/100 g freeze-dried yolk), whereas the 3% (feed +3% microalgal blend) and 10% (feed +10% *M. gaditana*) groups showed 2.7-fold (761.28 mg/100 g freeze-dried yolk) and 3.4-fold (956.67 mg/100 g freeze-dried yolk) increases, respectively (Table 5).

In this context, the study by Wu et al. [68] using *Nannochloropsis* sp. (NS) demonstrated that a 4% dietary inclusion over 48 days resulted in a DHA deposition of 89.79 mg/yolk, equivalent to 666.1 mg/100 g yolk. This represents a 2.1-fold increase compared to the control (42 yolk mg/yolk equivalent to 316.6 mg/100 g yolk). This trend is consistent with the results observed in the present study at a 3% supplementation level (feed +3% microalgal blend), indicating a comparable efficiency in DHA enrichment at moderate inclusion levels (Table 6).

On the other hand, when the diet was supplemented with 8% NS, DHA levels reached 835.3 mg/100 g yolk, representing a 2.6-fold increase compared to the control. A similar trend was observed at the 10% supplementation level in the present study. Taken together, these findings suggest that although DHA deposition increases with dietary inclusion, the efficiency of conversion and transfer to the yolk may not follow a linear pattern. Instead, it appears to approach a saturation threshold at higher inclusion levels (≥8%), where further increases in supplementation result in diminishing returns in yolk DHA deposition.

In addition to inclusion level, the lipid source also plays a key role in determining DHA deposition efficiency. Diets supplemented with *Schizochytrium limacinum* over a 42-week period showed a progressive increase in DHA content with increasing inclusion levels, reaching up to 194.04 mg/100 g yolk at 1.5% inclusión [71]. Similarly, Ao et al. [72], reported that graded supplementation with dehydrated whole-cell microalgae (*S. limacinum*) over a 32-week period significantly increased yolk DHA content, further supporting the effectiveness of this microalgal source (Table 6).

Additionally, results obtained with *Chlorella vulgaris* and *Spirulina platensis* further emphasize the critical role of microalgal species in determining DHA deposition efficiency. Although both treatments resulted in detectable DHA levels (196.25 and 118.02 mg/100 g yolk, respectively), these values remained markedly lower than those reported in our study at a 1% inclusion level of the microalgal blend, as well as those observed in other studies summarized in Table 6. This discrepancy highlights that DHA enrichment is not solely dependent on inclusion level, but is strongly influenced by the biochemical nature of the lipid source. In particular, *Chlorella* and *Spirulina* are not naturally rich in DHA, but predominantly contain shorter-chain n-3 fatty acids, which require enzymatic elongation and desaturation prior to deposition. Given the limited capacity of laying hens for these metabolic conversions, the efficiency of DHA transfer to the yolk is inherently constrained. Therefore, these findings reinforce the concept that microalgal sources inherently rich in preformed DHA are more effective for targeted yolk enrichment, underscoring the importance of selecting appropriate lipid sources over simply increasing dietary inclusion levels.

By comparison, fish oil supplementation resulted in a more moderate increase, with DHA values ranging from 142.66 to 152.24 mg/100 g yolk across comparable inclusion levels [71]. These results indicate that *S. limacinum* is a more efficient source of DHA for yolk enrichment, particularly at higher inclusion levels. This enhanced efficiency may be attributed to its biochemical composition, as *Schizochytrium* is naturally rich in DHA and provides it in lipid forms that are more readily absorbed and incorporated into egg yolk lipids (Table 6).

In contrast, fish oil contains a more complex mixture of fatty acids, which may limit the selective deposition of DHA due to metabolic competition among lipid classes. In this regard, Ryckebosch et al. [73], reported that DHA in microalgal oil is predominantly associated with glycolipids and phospholipids, facilitating more efficient absorption and utilization, whereas DHA in fish oil is mainly present in neutral lipids, which may reduce its bioavailability and subsequent deposition efficiency in egg yolk.

Consistent with this, Feng et al. [74], reported that although fish oil supplementation resulted in higher EPA deposition (up to 190 mg/100 g yolk), DHA levels showed a less pronounced increase compared to microalgal oil at equivalent inclusion levels. This suggests that while fish oil provides a broader spectrum of n-3 fatty acids, microalgal sources may offer a more targeted and efficient strategy for DHA enrichment, particularly when the goal is to maximize DHA deposition in egg yolk (Table 6).

It is worth noting that the supplementation of laying hen diets with EPA derived from microalgal sources facilitates its more efficient bioconversion into DHA, subsequently enabling its deposition in egg yolks. This phenomenon has been attributed to the fact that the hen's metabolism preferentially converts ALA into DHA rather than into EPA, thereby limiting the enzymatic conversion of the latter during the elongation and desaturation process [75]. In addition, the potential oxidation of intermediate fatty acids may also contribute to the enhanced deposition of DHA in egg yolks [76].

Furthermore, the concentration of arachidonic acid (C20:4 n-6, eicosatetraenoic acid) decreased in the eggs of supplemented hens, most likely as a consequence of a lower intake of n-6 precursors and/or due to metabolic competition with the newly incorporated n-3 fatty acids. In this context, the type of feed plays a fundamental role, since it has been reported that hens fed diets enriched with n-3 tend to exhibit a reduction in yolk ARA concentration [77]. A similar phenomenon was observed in our study, where the control pellets already provided n-3 through the contribution of ALA (70.49 mg/100 g feed; Table 3) and, once supplemented with microalgae, EPA was incorporated into the fatty acid profile (21.82 and 99.61 mg/100 g freeze-dried yolk for the 3% and 10%

Table 6
Comparative effects of microalgal and conventional lipid sources on EPA and DHA deposition and n-6:n-3 ratio in egg yolk.

Microalgae	% Inclusion	EPA (mg/100 g yolk)	DHA (mg/100 g yolk)	n3/n6 PUFA	Reference
Nannochloropsis sp.	0	Not detected	316.6	11.67	Wu et al. [68]
	1		399.6	10.11	
	2	Not detected	466.6	9.12	
	4		666.1	6.53	
	8	Not detected	835.3	4.41	
Schizochytrium limacinum	0.5	57.5	141.56	4.63	Kralik et al. [71]
	1	111.7	163.15	4.09	
	1.5	8.92	194.04	3.58	
	0.5	7.41	142.66	4.34	
Fish oil	1	6.48	148.91	4.34	
	1.5	4.44	152.24	3.8	
All-G-RichTM, Alltech Inc. (*S. limacinum*)	0	6.26	248	–	Ao et al. [72]
	1	9.26	509	–	
	2	–	717	–	
	3	–	776	–	
Microalgae oil	0	–	267	13.55	Feng et al. [74]
	0.125	0	720	5.2	
	0.25	9	1002	4.17	
	0.5	12	1189	3.66	
Fish oil	0	31	267	13.55	
	0.125	0	849	4.12	
	0.25	49	1008	3.5	
	0.5	96	1351	2.26	
S. limacinum	0	190	500	10.66	Kiran et al. [82]
	0.25	210	1040	14.46	
	0.50	230	1990	7.91	
	0.75	220	3130	5.21	
	1	230	4100	4.34	
Chlorella vulgaris	0	230	129.4	17.4	Panaite et al. [70]
	2	–	196.25	11.51	
Spirulina platensis	2	–	118.02	16.06	

treatments, respectively).

Similarly, linoleic acid (LA), the main dietary omega-6 fatty acid, decreased in yolk with increasing supplementation, from ~7.33 g/100 g yolk in the control group to ~5.81 g/100 g yolk at 10% inclusion (week 8; Table 5). This reduction may be explained by the well-described competition between LA and α-linolenic acid (ALA) for shared desaturation and elongation enzymes, particularly Δ6-desaturase [78]. Under these conditions, the increased availability of ALA may have limited the metabolic conversion of LA into long-chain n-6 PUFAs, while favoring the bioconversion of ALA into n-3 derivatives, contributing to the observed decrease in the n-6:n-3 ratio.

Interestingly, at week 8 of supplementation, EPA was detected in the yolks of hens fed 3% of the microalgal mixture (~24.5 mg/100 g yolk) and 10% *M. gaditana* (~51.0 mg/100 g yolk), while it remained undetectable in the control and 1% groups. This pattern may indicate a limited capacity for further elongation and desaturation at higher substrate availability, promoting EPA accumulation and its direct deposition in the yolk. This represents an added nutritional value, given the well-established bioactivity of EPA.

From a nutritional standpoint, the improvement in the fatty acid profile is highly relevant. Eicosanoids derived from omega-6 PUFAs (e.g., prostaglandin E2 and leukotriene B4 from arachidonic acid) act as more potent mediators of inflammatory and thrombotic responses than those derived from omega-3 fatty acids (e.g., prostaglandin E3 and leukotriene B5 from EPA) [79]. Consequently, an elevated omega-6/omega-3 ratio is strongly associated with proinflammatory and prothrombotic conditions, contributing to the development of chronic diseases such as atherosclerosis, cardiovascular diseases, autoimmune disorders, obesity, and diabetes [78,79].

Over the past century, this imbalance has been exacerbated by changes in dietary patterns, increasing from an approximate 1:1 ratio to values exceeding 15:1 in Western diets [48,80,81], largely due to the high intake of omega-6-rich vegetable oils and grain-fed animal products. This situation is reflected in the control eggs of the present study, which exhibited a high n-6:n-3 ratio typical of conventional corn–soybean-based diets.

In contrast, microalgal supplementation significantly improved the lipid profile of the eggs, increasing n-3 PUFA deposition (up to 1158.03 mg/100 g yolk) and reducing the n-6:n-3 ratio from 24:1 in the control group to 6:1 at 10% inclusion.

When compared with previous studies using microalgal biomass and conventional lipid sources, these results demonstrate a strong capacity for both DHA enrichment and modulation of the n-6:n-3 ratio, highlighting the effectiveness of microalgae as a functional and sustainable feed ingredient for the production of value-added eggs (Table 6).

This scenario is also reflected in the control eggs from the present study, originating from hens fed with conventional commercial diets based on corn and soybeans, which naturally exhibit a high n-6: n-3 ratio typical of current intensive production systems. In this context, the results obtained support the use of microalgae as functional and sustainable ingredients in poultry feed, enabling the production of omega-3–enriched eggs with potential health benefits for consumers and added value for the functional food market.

3.5. *Analysis of total carotenoid and lutein content in egg yolks from diets with different microalgae-supplemented pellets*

Regarding the results obtained for total carotenoids in each representative sample derived from 30 lyophilized yolks per treatment, it was observed that all groups receiving microalgae-supplemented feed exhibited an increase in total carotenoid content over time, with a notable accumulation in the 3% (microalgal mix of *Muriellopsis* sp. + *M. gaditana*) treatment with ~7.7 (mg carotenoids/100 g lyophilized yolk) and the 10% (*M. gaditana*) treatment with ~9.1 (mg carotenoids/100 g lyophilized yolk) (Fig. 6 A). These findings showed, by week 8, a 2.26-fold and 2.67-fold increase relative to the control, respectively. Moreover, the accumulation of carotenoids in the yolks was progressive as long as the supplemented diet was maintained.

These findings align with those reported by Papadopoulos et al. [83] and Failla et al. [84], who highlighted that carotenoids are fat-soluble compounds and, therefore, their bioavailability may be influenced by the quantity and quality of dietary fat included in feed formulations. In this context, the balance between saturated and unsaturated fatty acids observed in the microalgae-supplemented feed may serve as a relevant criterion for optimizing the bioavailability of these compounds in poultry diets, thereby underscoring the importance of adjusting dietary lipid composition to maximize the effective transfer of carotenoids to the egg yolk.

Lutein analyses in representative samples of lyophilized yolks from each supplementation treatment showed a significant increase in this carotenoid in the yolks corresponding to the 3% (microalgal mix of *Muriellopsis* sp. + *M. gaditana*) treatment, with values of 0.028% *w*/w lutein in lyophilized yolk samples. This result is consistent with the type of supplementation, as *Muriellopsis* sp. is a microalga rich in lutein [34]. In contrast, the 10% supplementation group exhibited a lower lutein

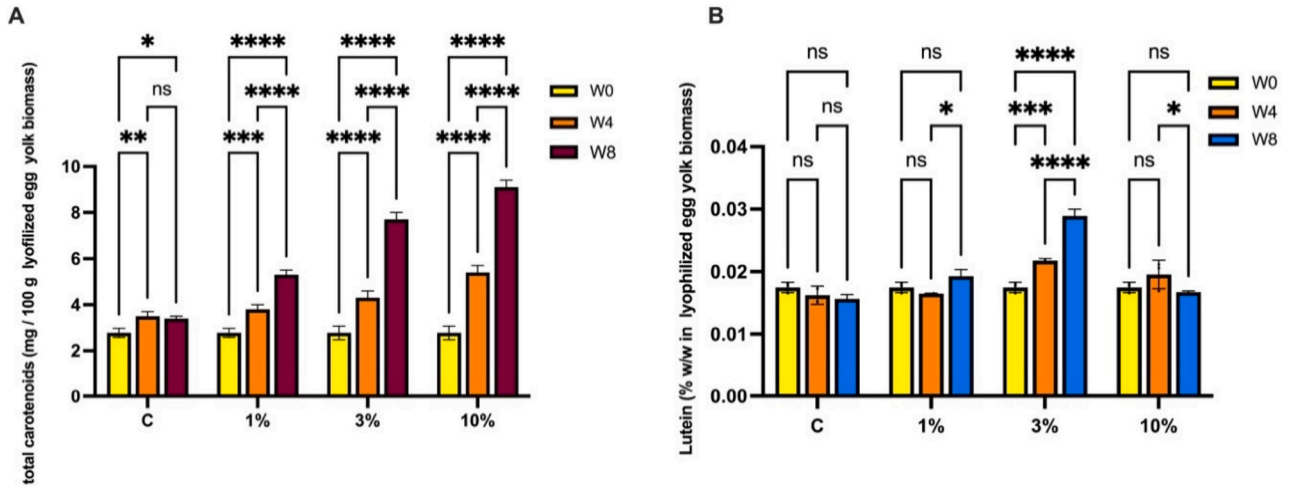


Fig. 6. Total Carotenoid and Lutein Content in Lyophilized Egg Yolks. A) Total carotenoid content (mg carotenoids/100 g lyophilized yolk). B) Lutein content (% weight/weight in lyophilized yolk samples). All analyses were conducted on a representative sample obtained from 30 lyophilized egg yolks. *Significantly different from control ($p < 0.05$) Values are based on the mean ± S.D. $n = 3$.

content, as *M. gaditana* is not primarily rich in this carotenoid [85]. Overall, these results demonstrated a dose-dependent response based on the microalgal content in each diet, as well as an effective transfer of carotenoids to the egg yolk. Nevertheless, previous studies, such as Golzar et al. [86], indicated that the linear relationship between dietary lutein levels and lutein deposition in eggs may reach a plateau beyond a certain dietary concentration. In that study, supplementing the diet with 250 mg/kg of lutein resulted in a significant increase in yolk lutein content, with the highest concentration observed at 750 mg/kg lutein. However, no significant differences in yolk lutein content were found between the 500 and 750 mg/kg supplementation levels.

Although the present study demonstrated efficient lutein transfer from microalgal biomass to egg yolks, the mechanisms underlying lutein absorption, metabolism, and deposition in laying hens remain poorly understood. Future studies should therefore investigate the physiological and metabolic pathways involved by incorporating intestinal histomorphological analyses, nutrient digestibility assessments, and blood serum kinetic studies. Such approaches would provide a more comprehensive understanding of lutein bioavailability and transport, helping to optimize dietary strategies for the production of lutein-enriched eggs.

3.6. *Physiological and biochemical parameters in laying hens fed with microalgae-supplemented pellets*

Overall, hens maintained normal biochemical parameters without signs of metabolic stress (Table 7). Regarding the physiological and biochemical parameters obtained from blood samples collected at weeks 4 and 8 of the microalgae-supplemented feeding trial, plasma liver enzymes such as AST, ALT, and GGT are recognized as valuable biomarkers in veterinary medicine. However, their clinical interpretation requires consideration of their high individual variability and sensitivity to non-pathological factors such as diet, stress, management, and physical activity [77,87]. In the present study, AST levels remained within the physiological range without significant variation between weeks 4 and 8, suggesting hepatic metabolic stability in all birds, consistent with the findings of Hrabcakova et al. [87] during the laying period.

With respect to ALT levels, slight increases were observed in some groups; however, these values remained within the reference range, indicating the absence of relevant hepatic damage. This pattern agrees with the observations of Viktorova et al. [88], who interpreted similar changes as favorable metabolic adaptations associated with the use of dietary additives. On the other hand, in the 10% (feed + *M. gaditana*) group, a marked increase in GGT levels was detected, along with a slight elevation in the other groups, including the control.

Since GGT may rise in response to oxidative stress or subclinical hepatic damage [77], its relative increase, in the absence of elevation of other hepatic enzymes, would not indicate potential hepatic injury. Instead, it may be associated with the physiological activation of hepatic metabolism during the laying phase, when lipid and protein synthesis intensifies for egg formation, which could explain the mild increase in these parameters. Overall, the variations observed in Table 7 appear to align with normal physiological adaptations during the laying cycle rather than toxic effects, supporting the safety of the evaluated supplements in Hy-Line Brown® hens.

Furthermore, the elevated calcium levels are attributed to the basal layer diet, which is formulated with high amounts of calcium carbonate to meet the intensive production demands of laying hens. Pelicia et al. [89], also described the correlation between calcium intake and eggshell strength, as well as the proportional relationship between blood calcium concentration and dietary calcium levels in laying hens. In summary, the variations reported in Table 7 are consistent with normal physiological adaptations throughout the laying cycle, rather than indicative of toxic effects, confirming the safety of the tested supplements in Hy-Line Brown® hens and supporting the use of enriched pellets as a safe and effective nutritional strategy for the production of high-quality functional eggs.

These results allow us to demonstrate that, although microalgae supplementation in laying hens has been previously explored, this study provides a novel and integrated approach based on a specifically designed microalgal biomass to achieve simultaneous enrichment of eggs with omega-3 fatty acids and antioxidant compounds, particularly lutein. In contrast to previous studies focused on single-species supplementation or single nutritional targets, this work adopts a comprehensive biotechnological strategy aimed at producing value-added eggs with enhanced functional properties, combining improved nutritional profiles with increased antioxidant capacity. Furthermore, the incorporation of microalgae into poultry diets offers distinct health-related benefits, including the enrichment of eggs with omega-3 fatty acids and carotenoids. Considering the growing consumer demand for healthier and more sustainable foods, this approach shows strong potential for the development of premium niche markets, where consumers are willing to pay a higher value for functional and nutritionally enhanced products [53], thereby reinforcing the role of microalgae as a key ingredient in future functional poultry production.

4. Conclusions

The inclusion of *M. gaditana* and *Muriellopsis* sp. biomass in laying hen diets is established as a viable and efficient strategy to enhance the nutritional value of eggs, particularly through enrichment in lutein and long-chain omega-3 polyunsaturated fatty acids (DHA and EPA). Supplementation with 3% of the microalgal mix produced a significant and progressive increase in yolk lutein content, reaching a 2.26-fold rise compared to the control, demonstrating the high bioavailability of microalgal pigments.

Additionally, increased DHA levels and the exclusive presence of EPA were observed in the 3% and 10% supplemented groups, accompanied by a substantial improvement in the omega-3/omega-6 ratio

Table 7
Physiological parameters of laying hens.

Parameter	Reference value	Control		1% (feed + microalgal mix)		3% (feed + microalgal mix)		10% *M. gaditana*	
		Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Glucose	200–286	286.8	244.25	278	267	278	249.75	286.5	244
Urea nitrogen	1–3	6	9.75	5.62	7	4.85	6.75	6.5	8.2
Creatinine	0.2–0.4	0.3	0.2	0.26	0.2	0.35	0.225	0.3	0.24
Total proteins	3.4–5.6	4.7	4.925	4.86	4.8	4.675	5.175	4.8	5.38
Albumin	1.5–2.1	1.64	2.025	1.78	1.9	1.85	1.85	1.75	2.04
Calcium	8.6–20.2	27.42	32.82	28.5	29.75	26.4	32.95	30.47	32.16
Phosphorus	2.7–6.9	5.34	6.05	4.68	4.7	4.35	5.8	4.95	6.02
Total bilirubin	0.1–0.5	0.28	2.075*	0.38	0.15	0.675	0.4	0.425	0.26
Alkaline phosphatase	55–1527	709.4	344.5	801.8	388.5	719.35	238.5	620.25	274.2
GOT (ALT) transaminase	147–245	169	144.5	180.4	210	183.73	184	169.5	188
GTP (ALT) transaminase	0–7	4	4	3.4	6	3	3.25	3.25	4.4
GGT	18–38	34.2	40.25	42.4	41	36.75	39	37.25	46

(from 1:18 in the control to 1:7 and 1:6, respectively), approaching the optimal dietary ratio recommended for human health. These findings reinforce the potential of microalgal supplementation to produce eggs with a more balanced and functional lipid profile. However, the 10% *M. gaditana* group did not exhibit proportional increases in fatty acid content, suggesting a possible saturation threshold in their transfer to the yolk, thereby identifying the 3% inclusion level as nutritionally and economically optimal. Finally, microalgae-enriched feed did not produce adverse effects on egg physical parameters or on the metabolic and hepatic profiles of laying hens, confirming the safety and physiological stability of the supplementation.

In conclusión, the results demonstrate that the use of specifically designed microalgal biomass enables the dual enrichment of eggs with omega-3 fatty acids and antioxidant compounds, particularly lutein, representing an advancement over previous approaches focused on single-species supplementation or isolated nutritional targets. This biotechnological strategy allows the production of value-added eggs with enhanced functional properties, combining improved nutritional quality with increased antioxidant capacity. Overall, these findings highlight the potential of microalgae-enriched pellets as a sustainable, safe, and nutritionally effective strategy for the production of functional eggs with added value for human health and the poultry industry. In this context, the enrichment of eggs with omega-3 fatty acids and carotenoids represents a promising approach for incorporation into premium niche markets for functional and value-added foods.

CRediT authorship contribution statement

Daniela Diaz Mac Adoo: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **María Teresa Mata:** Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria P. Kaltsidi:** Writing – review & editing, Writing – original draft. **Paula Ardiles:** Writing – original draft, Methodology. **Niel Jensen:** Methodology. **Victoria Cruz-Balladares:** Methodology, Investigation. **Henry Cameron:** Methodology. **Vladimir Avalos:** Methodology. **Francisco Sepúlveda-Sánchez:** Methodology. **Jennifer Palma:** Methodology. **Carlos Riquelme:** Writing – review & editing, Supervision, Project administration.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniela Diaz Mac-Adoo reports financial support was provided by National Agency for Research and Development. Reports a relationship with that includes:. Has patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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