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Multilevel Characterization of Eggs from Laying Hens Fed Dried *Haematococcus pluvialis* Biomass: Natural Biofortification, Lipid Modulation, and Instrumental Sensory Assessment

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Abstract

This study evaluated the effects of dietary supplementation with dried *Haematococcus pluvialis* biomass on egg quality in laying hens using a multilevel analytical approach. A total of 100 ISA Brown hens were divided into two groups: a control group (CTRL) fed a basal diet and an experimental group (HP) receiving the same diet supplemented with 0.075% *H. pluvialis*. Supplementation did not significantly affect most physical egg parameters, although yolk index and yolk height were improved in the HP group. A marked increase in yolk pigmentation was observed, with values reaching 15 on the DSM color fan compared to 8.4 in CTRL ($p < 0.0001$). Significant enhancements in yolk nutritional quality were detected, including increased total carotenoids and the presence of astaxanthin exclusively in the HP group. Mineral composition was also markedly affected, with significant increases in essential elements such as Fe, Mg, Zn, I, and P in both albumen and yolk. The fatty acid profile was favorably modulated, showing a reduction in saturated fatty acids and an increase in monounsaturated fatty acids, along with improved nutritional indices (AI, TI, HH). Instrumental sensory analysis revealed clear discrimination between groups based on color (E-eye), while differences in volatile profiles (E-nose) were less pronounced. However, a reduction in oviposition rate and egg mass was observed in the supplemented group. Overall, the inclusion of *H. pluvialis* biomass represents an effective strategy for the natural biofortification of eggs, improving their nutritional and functional value.

Keywords: *Haematococcus pluvialis*; astaxanthin; laying hens; egg quality; carotenoids; fatty acid profile; electronic nose; electronic eye

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1. Introduction

In recent years, increasing attention from both consumers and the scientific community toward the sustainability of animal production systems has driven the development of innovative nutritional strategies aimed at improving the quality of animal-derived products while reducing environmental impact. In this context, the use of alternative or functional feed ingredients represents a promising approach not only to enhance sustainability but also to improve the qualitative and nutritional characteristics of the final products.

Eggs, due to their high nutritional value and versatility, have become a key target for such strategies. Among egg quality parameters, yolk color represents one of the most important sensory attributes influencing consumer perception and acceptance [1]. To enhance yolk pigmentation, carotenoids are commonly added to poultry diets. These fat-soluble pigments are absorbed in the intestine and deposited in the yolk during egg formation, determining its color. Beyond their technological role, carotenoids have been widely associated with beneficial effects on human health, including reduced risks of cataracts [2], cardiovascular diseases, certain cancers, and age-related macular degeneration [3]. Moreover, their antioxidant activity contributes to the mitigation of oxidative stress and atherosclerosis [4,5], as well as to the regulation of cellular processes [6]. As laying hens are unable to synthesize carotenoids endogenously, these compounds must be provided through the diet [7,8]. In recent years, increasing interest has been directed toward natural sources of carotenoids, particularly microalgae, which are characterized by a rich and diverse nutritional composition. Microalgae contain high levels of proteins, lipids, carbohydrates, minerals, vitamins, and bioactive compounds, including carotenoids [9,10]. They can grow in a wide range of environments, including freshwater, marine systems, and wastewater [11,12], making them highly attractive as sustainable feed resources. In addition, microalgae provide essential fatty acids [13], vitamins [14], minerals [15], and antioxidants [16], all of which contribute to a balanced poultry diet [17]. Among microalgal species, *Haematococcus pluvialis* is particularly noteworthy due to its high content of astaxanthin, a carotenoid with strong antioxidant activity and recognized benefits for both animal and human health. Astaxanthin is also a highly effective pigment capable of significantly influencing yolk color. Previous studies in aquaculture and poultry science have demonstrated the potential of astaxanthin supplementation in improving product quality [18–20]. However, most studies have focused on the use of purified astaxanthin, while limited information is available regarding the direct use of dried *H. pluvialis* biomass as a feed ingredient in laying hens. The use of whole microalgal biomass may represent a more sustainable and cost-effective alternative, as it provides not only astaxanthin but also a wide range of bioactive compounds that may act synergistically. Therefore, the inclusion of *H. pluvialis* in laying hen diets could contribute to the natural biofortification of eggs, potentially improving their lipid profile, mineral content, antioxidant properties, and overall nutritional value.

In addition to compositional aspects, sensory characteristics play a crucial role in determining egg quality and consumer acceptance. While yolk color is a primary visual attribute, aroma also significantly influences consumer preference and purchasing decisions [21]. Consumer preference for yolk color varies widely, with darker yolks often associated with higher perceived quality and market value [22,23]. Previous studies have shown that carotenoid supplementation can enhance yolk pigmentation without negatively affecting flavor or overall egg quality [24,25]. In this context, instrumental sensory analysis has gained increasing importance as an objective and reproducible approach for evaluating food quality. Technologies such as the electronic eye (E-eye) and electronic nose (E-nose) are designed to simulate human sensory perception, enabling the digitalization of visual and olfactory attributes [26]. These tools provide rapid and standardized measurements and can complement traditional analytical methods in food quality assessment. The application of artificial sensing systems in foods of animal origin has been widely reviewed, highlighting their potential for rapid quality evaluation and classification [27]. Their ability to support objective discrimination of food samples has been demonstrated in different studies based on multivariate data analysis approaches [28]. Moreover, these technologies have proven effective in detecting subtle quality differences that may not be captured by conventional analyses [29,30].

The present study aimed to evaluate the effects of dietary supplementation with dried *H. pluvialis* biomass on egg quality in laying hens, adopting a multilevel approach that integrates physical traits, detailed compositional and nutritional profiling, fatty acid composition, and instrumental sensory analysis (E-eye and E-nose). Particular attention was given to assessing the potential of whole microalgal biomass, as a natural source of astaxanthin, for the production of value-added and nutritionally enhanced eggs. This study also explores whether the use of *H. pluvialis* biomass can represent a sustainable and effective strategy for egg biofortification without compromising standard quality traits.

2. Materials and Methods

2.1. Experimental

All experimental procedures were approved by the Ethics Committee of the University of Messina (Messina, Italy) (Prot.24/2025, approved on 30 March 2025).

A total of 100 ISA Brown laying hens, 20 weeks of age, were used in this study and randomly allocated into two experimental groups. The animals used in this study were reared in a commercial poultry farm (Azienda Agricola Tomarchio, Italy) under standard production conditions. The hens were housed under controlled environmental conditions with standardized hygiene and temperature management. No adverse events were observed during the experimental period. Each group consisted of 50 hens arranged into 10 replicates, with each replicate comprising 5 hens housed in enriched cages measuring 102 cm × 52 cm × 45 cm (length × width × height). The available floor space was approximately 1061 cm^2 per hen. Cages were equipped with perches, nest boxes, a litter area for foraging behavior, claw-shortening devices, and feeding and watering systems meeting the requirements for enriched cages under the relevant animal welfare legislation. No a priori sample size calculation was performed. The experimental trial lasted 60 days. The experimental period was deliberately scheduled during the early laying phase to evaluate the initial transfer and deposition of bioactive compounds from dietary *H. pluvialis* biomass into eggs during a phase of active physiological adaptation and egg formation. During this period, hens were provided with a fixed daily feed allowance of 130 g per bird.

All birds were fed a standard basal diet during a one-week adaptation period. After this period, the control group (CTRL) continued to receive only the basal diet, whereas the experimental group (HP) was fed the same basal diet supplemented with 0.075% dried algal biomass. The inclusion level of 0.075% dried *H. pluvialis* biomass was selected to reflect a commercially realistic supplementation strategy aimed at providing natural astaxanthin without substantially altering diet composition or nutrient balance. All animals had ad libitum access to water. The diets were formulated to meet the nutritional requirements of laying hens formulated according to NRC (1994) [31] requirements and ISA Brown management guidelines.

The ingredients and nutritional composition of the basal diet are reported in Table 1.

Table 1. Formulation and chemical composition of the experimental diets for the control (CTRL) and *Haematococcus pluvialis* supplemented group (HP).

Ingredients (%)	CTRL	HP
Corn	54.80	54.7
Soybean meal	21	21
Calcium carbonate	9	9
Sunflower meal	9	9
Soybean oil	2.50	2.5

Table 1. *Cont.*

Ingredients (%)	CTRL	HP
Dicalcium phosphate	1.50	1.50
Sugar cane molasses	1	1
Sodium chloride	0.50	0.50
Vitamin and mineral premix [1]	0.50	0.50
Sodium bicarbonate	0.15	0.15
Dehydrated algae (HP)	-	0.075
Proximate analysis, (g/100 g as fed)		
Moisture	9.05	10.19
Ash	14.00	14.00
Fat	16.41	17.03
Protein	16.44	17.08
Crude fiber	3.30	3.30
Calcium	3.90	3.90
Phosphorus	0.55	0.55
Sodium	0.18	0.18
Methionine	0.42	0.42
Lysine	0.85	0.85
Total Carotenoids (mg/kg)	15.56	32.52
Total polyphenols (mg/kg)	218.41	263.05
Vitamin E (mg/kg)	24.03	24.50

[1] Supplied per kg of feed: Vitamin A: 10.000 IU, Vitamin D3: 2000 IU, Vitamin D: 1200 IU, Vitamin E: 25 mg, Vitamin K3: 3 mg, Vitamin B1: 3.5 mg, Vitamin B2: 7.5 mg, Vitamin B6: 4 mg, Vitamin B12: 0.03 mg, Biotin: 0.06 mg, Niacin: 50 mg, Calcium D-pantothenate: 13 mg; Folic acid: 1 mg; betaine hydrochloride: 163 mg; choline chloride: 170 mg; Fe (Iron sulfate (II) monohydrate): 50 mg; Mn (Manganese(II) oxide): 85 mg; Zn (Zinc oxide): 65 mg; Cu (Copper(II) sulfate pentahydrate): 8.5 mg; I (Potassium iodide): 1.50 mg; Se (Sodium selenite): 0.2 mg.

2.2. Productive Performance of Laying Hens

Data related to productive performance were collected and recorded throughout the experimental period. Oviposition rate and egg weight were recorded daily. Hen body weight was measured at the beginning and at the end of the trial. Productive performance parameters included oviposition rate, egg weight, and egg mass. Egg mass, an indicator of overall egg output that combines egg production and egg weight, was calculated as the product of the egg production rate and the average egg weight. Feed intake was controlled by providing a fixed daily feed allowance (130 g/hen), and no feed refusals were observed during the experimental period.

2.3. Sample Collection and Physical Parameters of Eggs

At the end of the experimental period, a total of 200 eggs were collected for the evaluation of their physical, chemical, and nutritional characteristics.

Initially, the individual weight of each egg was determined using an electronic balance (Mettler-ToledoPL 3000, International Inc., Greifensee, Switzerland, accuracy 0.1 g), while simultaneously performing a visual inspection to identify potential microcracks or shell damage.

Morphometric measurements were then conducted using a digital caliper (Stainless Hardened, Wenzhou Shengce Instrument Co., Ldt, Wenzhou, China, accuracy 1 mm), recording the longitudinal height and equatorial diameter of each egg. Egg circumference was measured using a flexible measuring tape (Stanley Black & Decker Italia, S. r. l., Vimercante (Mb), Italy).

Following morphological characterization, 80 eggs were manually opened and separated into the three main fractions: albumen, yolk, and shell. Each component was weighed individually to determine its percentage relative to the total egg mass.

Eggshells were dried in a ventilated oven at 60 °C for 12 h to remove residual moisture and allow accurate weighing for the calculation of shell percentage relative to the fresh egg weight.

The remaining eggs were used for freshness and quality parameter analysis. The following measurements were performed using a TA.XTPlusC texturometer (Stable Micro Systems Ldt,. Godalming, Surrey, United Kingdom): eggshell breaking strength (N), eggshell deformation (mm), shell weight (g), shell thickness (mm), albumen height (mm), Haugh units, yolk membrane strength (N), yolk height (mm), yolk index, yolk width (mm), and air cell height (mm). Yolk color was determined using the DSM colorimetric scale, also known as the "Yolk Color Fan" or "Roche scale".

For the determination of nutritional parameters, a total of 30 defect-free eggs per group were collected (n = 30 per group). Eggs were pooled in sets of three to obtain composite samples, resulting in 10 pooled samples per group (n = 10). For each pooled sample, albumen and yolk were separately collected and homogenized. The resulting samples were stored in dark vials at +4 °C until further analysis. Only defect-free eggs were included in the analysis; no additional exclusions of samples or data points were performed.

2.4. Chemical Analysis of Eggs

2.4.1. Materials and Reagents

For the proximate composition, the Kjeldahl catalyst was supplied by Carlo Erba (Milan, Italy).

For the elemental analysis, the following reagents were employed: nitric acid (65% *v*/*v*) and hydrogen peroxide (30% *v*/*v*), with trace metal analysis grade, and ultrapure water, with resistivity of 10 mΩ cm (J.T. Baker, Milan, Italy). Stock solutions of commercial standards of Na, Mg, K, Ca, P, Fe, Cu, Mn, Zn, Se, As, Cd, and Pb (1000 mg/L in 2% HNO_3) were from Fluka (Milan, Italy). For the study of the FA composition, n-heptane and n-hexane (reagent grade) were purchased from J.T. Baker. Fatty acid methyl esters (FAMEs) reference standards (C4–C24) were supplied by Supelco (Bellefonte, PA, USA).

For the determination of astaxanthin, total carotenoids and tocopherols: acetone (reagent grade), n-hexane and ethyl acetate (HPLC-grade), water and acetonitrile (LC-MS grade) were supplied by J.T. Baker and LiChrosolv (Merk, Darmstadt, Germany). Reference standards of single tocopherols (i.e., α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol, 98% purity each), astaxanthin (98% purity), and β-carotene (≥95% purity) were purchased from Supelco.

2.4.2. Proximate Composition

The proximate composition of yolk and albumen samples was determined in terms of moisture, protein and lipid content according to the AOAC official protocols of analysis [32]. Specifically, moisture was assessed according to the AOAC method 925.30. Crude protein was determined by the Kjeldahl method, as reported in the AOAC method 925.31; while for the total lipid content, the AOAC method 925.32 was used.

Every nutritional parameter was analyzed in six analytical replicates, along with suitable analytical blanks.

2.4.3. Inorganic Elements

The determination of inorganic elements occurred by following the protocol of analysis already proposed by Albergamo et al. [33].

Briefly, each albumen and yolk sample (0.5 g, fresh weight) was mineralized with 7 mL of HNO_3 and 1 mL of H_2O_2 by the microwave digestion system Ethos 1 (Milestone, Bergamo, Italy) according to the following temperature program: 0–200 °C in 10 min (step 1), 200 °C held for 10 min (step 2), at a constant microwave power of 1200 W. After cooling

down to room temperature, the digested sample was diluted up to 25 mL with ultrapure water. A quadrupole ICP-MS iCAP Q (Thermo Scientific, Waltham, MA, USA) was used for the analysis of minerals (i.e., Na, K, Mg, Ca, and P), trace essential (i.e., Mn, Fe, Cu, and Zn), and non-essential or potentially toxic (i.e., As, Cr, Ni, Cd, and Pb) elements.

The operating parameters were incident radio frequency (rf) power 1500 W; plasma gas flow rate [argon (Ar)] 14 L/min; auxiliary gas flow rate (Ar) 0.8 L/min; carrier gas flow rate (Ar) 1.10 L/min. The instrument was operated with helium (He) as collision cell gas (4.7 mL/min) and was equipped with a spray chamber set at 2.7 °C. The injection volume and the sample introduction flow rate were equal to 200 µL and 0.93 mL/min, respectively. Spectra acquisition occurred in full scan mode (dwell time 0.5 or 0.01 s/point, based on the analyte).

All samples were screened in triplicate along with analytical blanks and data acquisition occurred through Qtegra™ Intelligent Scientific Data Solution (Thermo Scientific™). For quantification, a six-point calibration curve was built up for each analyte (r2 ranging from 0.9991 (Mg) to 0.9998 (Pb)). Six replicate measurements along with analytical blanks were carried out for every sample, to ensure the reproducibility of experimental results and exclude potential interferences between analysis.

2.4.4. Fatty Acid (FA) Composition

Due to the negligible amount of lipids in egg albumen, only yolks from CTRL and HP eggs were included in the study of FA composition.

Hence, lipid extracts from yolks were subjected to a cold transesterification consisting of a sample dilution with 2 mL of n-heptane and mixing with 200 µL of a methanolic KOH solution. The obtained mixtures was centrifuged at 8000 rpm for 10 min and the recovered supernatants, containing the FAMEs, were filtered and injected into a gas chromatograph (GC) equipped with a split/splitless injector and coupled to a flame ionization detector (FID) (Dani Master GC1000, Dani Instrument, Milan, Italy).

For the analysis of FA composition, the protocol proposed by Albergamo et al. [33] was applied, with slight modifications. Specifically, the chromatographic analysis was carried using a Supelco SLB-IL100 capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm, Supelco, Sigma Aldrich, St. Louis, MO, USA), and with the following operating conditions: column oven temperature from 50 (hold time 2 min) to 250 °C (hold time 15 min) at 2 °C/min; injector and detector temperatures both at 250 °C; carrier gas (He) at a linear velocity of 30 cm/s (constant), and with an initial head pressure of 99.5 Kpa. The makeup gas (He), hydrogen (H_2), and air flows were set at 25 mL/min, 40 mL/min, and 280 mL/min, respectively. The injection volume was 1 µL, with a split ratio of 1:50. Data acquisition and management were performed using Clarity Chromatography Software v. 4.0.2 (DataApex, Prague, Czech Republic).

All samples were analyzed in six replicates, along with analytical blanks (n-heptane), to exclude any interferences of the solvent or potential carry-over effects from one sample to the next. The retention times of FAMEs of nutritional relevance were compared with those of reference standards. Then, the quantification occurred by calculating their individual percentages in relation to the total chromatogram area. The following indices were calculated to evaluate the nutritional quality of the yolk lipid fraction:

Atherogenic index (AI) (1) and thrombogenic index (TI) (2) as per Ulbricht and Southgate [34]:

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0)/(\sum MUFA + \sum PUFA) \quad (1)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0) \,/\, [0.5 \times \sum MUFA + 0.5 \times \sum n\text{-}6 + 3 \times \sum n\text{-}3 + (n\text{-}3/n\text{-}6)] \quad (2)$$

In Equations (1) and (2), ΣSFA represents the sum of saturated fatty acids; ΣMUFA represents the sum of monounsaturated fatty acids; ΣPUFA represents the sum of polyunsaturated fatty acids; Σn-3 represents the sum of omega-3 fatty acids; Σn-6 represents the sum of omega-6 fatty acids. All fatty acids were expressed as percentage of total fatty acids.

Hypocholesterolemic/hypercholesterolemic ratio (HH), (3) according to Santos-Silva et al. [35]:

$$HH = (\sum C18:1 + \sum PUFA)/(C14:0 + C16:0) \quad (3)$$

Omega-6/Omega-3 ratio (n-6/n-3) (4)

$$n\text{-}6/n\text{-}3 = \sum (n\text{-}6 \text{ fatty acids})/\sum (n\text{-}3 \text{ fatty acids}) \quad (4)$$

Peroxidation index (PI), (5) according to Arakawa and Sagai [36]:

$$PI = (0.025 \times \%monoenes) + (1 \times \%dienes) + (2 \times \%trienes) + (4 \times \%tetraenes) + (6 \times \%pentaenes) + (8 \times \%hexaenes) \quad (5)$$

2.4.5. Measurement of Vitamin E in Yolk

Due to a lipophilic nature, vitamin E, intended as sum of α- β- γ- and δ-tocopherols, is primarily deposited in the yolk of an egg, and not the albumen. Consequently, vitamin E was assessed only in yolk samples. Tocopherols isomers were determined by following the protocol described by Lo Turco et al. [37]. Specifically, each yolk lipid extract (0.5 g) was dissolved in 2 mL of n-hexane and analyzed by a high-performance liquid chromatography system with fluorescence detection (HPLC-FD, Prominence HPLC System with RF-20A detector, Shimadzu, Milan, Italy). Separation was achieved on a LiChrosorb Si60 column (250 mm × 4.6 mm, 5 µm, Merck, Darmstadt, Germany) with a matching guard column, maintained at 40 °C. The mobile phase consisted of n-hexane/ethyl acetate (90:10, *v*/*v*) under isocratic conditions at a flow rate of 0.8 mL/min. The identification of tocopherols was carried out by a direct comparison with the retention time of relative commercial standards at respective excitation and emission wavelengths of 295 nm and 330 nm. The quantitative analysis was performed by constructing appropriate external calibration curves for every investigated tocopherol. All samples were analyzed in six replicates, along with analytical blanks (n-hexane).

2.4.6. Measurement of Total Carotenoids in Yolk

Total carotenoids were assessed by following the AOAC method 958.05 with slight modifications [38]. Specifically, each yolk sample (4.0 g, fresh weight) was mixed with acetone in two steps: first, 1 mL was added to form a smooth paste, and then 49 mL were added to obtain a well-mixed solution. The solution was then filtered by using Whatman paper (Cytiva, Marlborough, MA, USA), and the filter was washed with 10 mL of acetone. The absorbance of the extract recovered in acetone was read in an UV spectrophotometer (UV-2401 PC; Shimadzu, Milan, Italy) at 450 nm. For quantification purposes, an external calibration curve was constructed with the commercial standard of β-carotene and, consequently, total carotenoids were expressed as mg of β-carotene equivalents per kg of sample. All samples were analyzed in six replicates, along with analytical blanks (acetone).

2.4.7. Measurement of Astaxanthin in Yolk

The same extracts used for the determination of total carotenoids were employed for the determination of astaxanthin in yolk samples. To this purpose, an analytical protocol based on ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was optimized and validated with the aid of the commercial standard of astaxanthin. The analysis were performed on a Shimadzu Prominence UFLC

XR system (Shimadzu, Kyoto, Japan) interfaced through an electrospray ionization (ESI) source to an LCMS-8040 triple quadrupole mass spectrometer (MS) (Shimadzu, Kyoto, Japan). Chromatographic separations occurred on a Acquity UPLC®® BEH C8 column (1.7 µm, 2.1 × 50 mm) from Waters (Wexford, Ireland). The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The gradient program was as follows: 0–1 min, 95–100% B, 1–4 min, 100% B. The system was re-equilibrated to the initial conditions (95% B) at 4.0 min. Oven temperature and injection volume were, respectively, set at 28 °C and 2 µL. The flow rate was 0.4 mL/min, and a stainless steel splitting device (VICI AG International, Schenkon, Switzerland) assured that only 300 µL/min of the flow were directed from the LC system to the ESI interface. The ionization source was used in positive ionization mode according to the following parameters: capillary voltage, 4.5 kV; heat block temperature, 400 °C; desolvation line (DL) temperature, 250 °C; drying gas flow (N2), 15 L/min; nebulizing gas flow (N2), 3 L/min; collision induced dissociation (CID) gas pressure (Ar), 230 kPa. The precursor ion [M−H]+ of astaxanthin was first full scanned over a m/z range of 100–700, with an event time of 0.309 s, scan speed: 7500 amu/s. Then, the precursor ion was selected for studying its multiple reaction monitoring (MRM) transitions. To this purpose, parameters, such as dwell time, Q1 pre bias, collision energy, Q3 pre bias, were automatically optimized by the instrumental software (v. 5.53 SP2, Shimadzu, Kyoto, Japan). The MRM transitions for astaxanthin were as follows: (m/z) 597.4 → 173.2 and (m/z) 597.4 → 147.1. These two most abundant product ions were selected and used respectively for qualification and quantification purposes. Data were acquired by LabSolution software (v. 5.53 SP2, Shimadzu, Kyoto, Japan). Six replicate measurements were performed for every sample.

2.5. Sensory Analysis

The organoleptic characteristics of the yolk were evaluated using an instrumental sensory analysis platform composed of an electronic eye (E-Eye) and electronic nose (E-Nose).

Visual analysis was performed using the Vision System (CVS), Iris Visual Analyzer 400 (Alpha MOS, Toulouse, France). A total of 10 images per group were acquired under controlled lighting conditions. Prior to analysis, yolks were carefully separated from the albumen and placed on a sample holder, which was then positioned on the acquisition surface inside the measurement chamber. Images were captured using the high-resolution camera integrated into the system. The images were processed using dedicated software to extract colorimetric variables. The resulting data were subsequently subjected to multivariate statistical analysis to identify potential differences among experimental groups.

Aroma analysis was performed using the FOX 4000 Electronic Nose (Alpha MOS, Toulouse, France). The system is composed of an array of metal oxide semiconductor (MOS) sensors sensitive to volatile compounds present in the sample headspace.

For analysis, 2 g of egg (yolk and albumen) were placed in sealed vials and incubated at 50 °C for 1200 s. Subsequently, the headspace was automatically aspirated and directed into the sensor chamber. Interaction between volatile compounds and the sensors induced changes in the electrical resistance of the gas sensors, generating characteristic signals for each sample. Sensor responses were recorded by the system and subsequently analyzed using multivariate statistical techniques.

2.6. Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) with the XLSTAT statistical software (version 2021.2.2, Addinsoft, New York, NY, USA), with dietary treatment considered as a fixed effect. For productive performance data, the replicate cage was considered the experimental unit (n = 10 per treatment).

Significant differences between group means were determined using Tukey's multiple comparison test, with a significance threshold set at $p < 0.05$.

Principal component analysis (PCA) was applied to results obtained from instrumental sensory analyses using AlphaSoft (version 2021.2.2, Alpha MOS, Toulouse, France) to explore patterns in the data and to reduce the dimensionality of variables. The PCA was performed on datasets derived from the E-eye and E-nose to evaluate discriminative ability among experimental groups. Principal components were extracted based on their ability to explain the maximum variance in the dataset, and results were interpreted by examining both score plots and loading plots. A discrimination index was also calculated to assess the degree of separation among groups.

Blinding was not applied during the experimental procedures; however, instrumental analyses were performed using objective measurement systems.

3. Results

3.1. Productive Performance of Laying Hens

Productive performance parameters are reported in Table 2. Initial and final body weights did not differ significantly between CTRL and HP groups ($p > 0.05$). Similarly, average egg weight was not affected by dietary treatment ($p > 0.05$).

Table 2. Productive performance of laying hens fed control (CTRL) and *H. pluvialis* supplemented diets (HP) at day 60 of the experimental period. Values are expressed as mean ± SD.

	CTRL	HP	*p*-Value
Initial weight (kg)	1.51 ± 0.10	1.52 ± 0.45	0.778
Final weight (kg)	1.63 ± 0.13	1.61 ± 0.16	0.554
Oviposition rate (%)	79 ± 0.09	71 ± 0.10	0.020
Egg weight (g)	52.01 ± 1.27	51.23 ± 1.88	0.192
Egg mass (g)	41.52 ± 5.21	36.51 ± 6.09	0.022

In contrast, oviposition rate and egg mass were significantly reduced in hens fed the *H. pluvialis* diet compared to the control group ($p < 0.05$).

3.2. Physical Parameters of Eggs

Dietary supplementation with *H. pluvialis* did not significantly affect most of the physical parameters of the eggs (Table 3). No significant differences were observed between CTRL and HP for egg weight, egg length, form index, shell weight, shell percentage, albumen weight, albumen percentage, yolk weight, and yolk percentage ($p > 0.05$). Significant differences were observed for egg width and egg circumference, which were higher in the CTRL group compared to HP ($p < 0.05$). Conversely, shell index, yolk height, and yolk index were significantly higher in the HP group ($p < 0.05$). Yolk color was significantly affected by dietary treatment, with higher values observed in the HP group compared to CTRL ($p < 0.0001$). No significant differences were found for shell breaking strength, shell deformation, shell thickness, albumen height, Haugh units, vitelline membrane strength, yolk width, and air cell height ($p > 0.05$).

Table 3. External and internal physical parameters of eggs from laying hens fed control (CTRL) and *H. pluvialis* supplemented diets (HP) at day 60 of the experimental period. Values are expressed as mean ± SD.

Parameters	CTRL	HP	*p*-Value
Whole egg weight (g)	53.44 ± 4.50	52.48 ± 3.63	0.130
Egg length (cm)	5.33 ± 0.19	5.32 ± 0.19	0.798
Egg width (cm)	4.19 ± 0.11	4.15 ± 0.10	0.020
Egg circumference (cm)	13.52 ± 0.41	13.35 ± 0.34	0.004
Form index (%)	78.71 ± 2.15	78.10 ± 2.54	0.103
Shell weight (g)	6.77 ± 0.66	7.15 ± 0.51	0.089
Shell (%)	12.75 ± 0.90	13.45 ± 0.75	0.793
Albumen weight (g)	33.67 ± 2.06	32.85 ± 2.07	0.905
Albumen (%)	63.28 ± 1.66	61.74 ± 2.31	0.904
Yolk weight (g)	12.95 ± 0.83	13.06 ± 0.72	0.967
Yolk (%)	24.36 ± 1.35	24.58 ± 1.37	0.965
Shell index (g/cm^2)	0.30 ± 0.02	0.32 ± 0.02	0.038
Shell breaking strength (N)	58.25 ± 3.97	54.88 ± 9.96	0.334
Shell deformation (mm)	0.79 ± 0.04	0.78 ± 0.07	0.659
Egg albumen height (mm)	12.56 ± 7.76	14.68 ± 4.28	0.458
Haugh unit	103.56 ± 25.11	115.88 ± 11.68	0.177
Shell thickness (mm)	0.38 ± 0.07	0.4 ± 0.02	0.216
Vitelline membrane strength (N)	0.18 ± 0.00	0.18 ± 0.00	0.172
Yolk color (1–15)	8.4 ± 1.58	15 ± 0.00	<0.0001
Yolk height (mm)	12.51 ± 0.96	13.8 ± 1.19	0.016
Yolk width (mm)	37.10 ± 1.33	37.14 ± 1.47	0.942
Yolk index	0.33 ± 0.03	0.37 ± 0.03	0.014

3.3. Nutritional Evaluation of Eggs

3.3.1. Albumen

With respect to the main nutritional characteristics (Table 4), dietary supplementation significantly affected the moisture and the protein content of the albumen. In fact, moisture was significantly higher in the HP group than in the CTRL group (86.42% vs. 87.422%, $p < 0.05$), while crude protein was significantly lower in experimental eggs than the control counterpart (12.50% vs. 11.54%, $p < 0.001$). However, lipid content did not differ significantly between groups (0.25% vs. 0.32%, $p > 0.05$). Similarly, total carotenoids of albumens were not significantly affected by the diet (1.66 mg BCE/kg vs. 1.78 mg BCE/kg, $p > 0.05$) (Table 4). Considering the profile of inorganic elements (Table 5), minerals were found in both study groups according to the following quantitative order: Na > K > P > Mg > Ca. The levels of these minerals spanned from 1598.78–3019.16 mg/kg (Na) to 83.55–153.51 mg/kg (Ca). The statistical analysis confirmed that the albumen from HP group had significantly higher amounts of all minerals than the albumens from the CTRL group ($p < 0.0001$). On the other hand, essential trace metals were revealed in the following quantitative sequence: Zn > Fe > Cu > I > Mn > Se, with contents varying between 3.24–5.80 mg/kg (Zn) and 0.03–0.04 mg/kg (Se). Statistically significant increases of Fe, Cu, Zn, and I were observed in albumens following dietary supplementation ($p < 0.0001$). However, Mn and Se were not only were found at the lowest levels, but also in amounts that were not statistically significantly different (Mn: 0.14 mg/kg vs. 0.15 mg/kg, $p > 0.05$; Se: 0.14 mg/kg vs. 0.15 mg/kg, $p > 0.05$) between the study groups. Investigated non-essential or potentially toxic trace elements were present at low levels (e.g., Ni and As at <0.02 mg/kg) or even not detected (i.e., Cr, Cd and Pb), in both CTRL and HP albumens. Nonetheless, significant different contents of Ni and As were highlighted by the statistical analysis ($p < 0.0001$) (Table 5).

Table 4. Main nutritional characteristics of egg albumen from laying hens fed CTRL and HP diets. Data are expressed on a fresh weight basis and as mean ± SD.

Nutritional Parameter	CTRL	HP	*p*-Value
Moisture (%)	86.42 ± 0.72	87.42 ± 0.31	0.011
Fat (%)	0.25 ± 0.08	0.32 ± 0.05	0.115
Protein (%)	12.50 ± 0.41	11.54 ± 0.22	0.000
Total carotenoids (mg BCE/kg)	1.66 ± 0.48	1.78 ± 0.42	0.658

Table 5. Profile of inorganic elements (mg/kg) of egg albumen from laying hens fed CTRL and HP diets. Data are expressed on a fresh weight basis and as mean ± SD.

Analyte	CTRL	HP	*p*-Value
Minerals (mg/kg)			
Na	1598.78 ± 9.48	3019.16 ± 38.80	<0.0001
K	1411.68 ± 2.81	3752.56 ± 35.84	<0.0001
Mg	127.02 ± 2.36	350.21 ± 2.98	<0.0001
Ca	83.55 ± 2.63	163.51 ± 3.81	<0.0001
P	148.91 ± 1.96	585.33 ± 4.18	<0.0001
Essential trace elements (mg/kg)			
Mn	0.14 ± 0.01	0.15 ± 0.01	0.038
Fe	0.92 ± 0.03	14.29 ± 0.66	<0.0001
Cu	0.49 ± 0.02	0.81 ± 0.02	<0.0001
Zn	3.24 ± 0.07	6.80 ± 0.15	<0.0001
Se	0.03 ± 0.01	0.04 ± 0.01	0.092
I	0.15 ± 0.01	0.88 ± 0.05	<0.0001
Non-essential or potentially toxic trace elements (mg/kg)			
Cr	0.00 ± 0.00	0.00 ± 0.00	-
Ni	0.00 ± 0.00	0.02 ± 0.01	<0.0001
As	0.01 ± 0.002	0.02 ± 0.00	<0.0001
Cd	0.00 ± 0.00	0.00 ± 0.00	-
Pb	0.00 ± 0.00	0.00 ± 0.00	-

3.3.2. Yolk

Table 6 shows the main nutritional parameters analyzed for the yolk samples. Moisture content in the yolk was not affected by the dietary treatment (48.22% vs. 48.64%, $p > 0.05$). Conversely, total lipids were significantly higher in experimental yolks than in the control ones (29.23% vs. 21.54%, $p < 0.0001$), whereas protein content of HP yolks was significantly lower compared to the CTRL counterpart (16.26% vs. 17.57%, $p < 0.0001$). As expected, total carotenoids in yolk were significantly higher following dietary supplementation with *H. pluvialis* (19.44 mg BCE/kg vs. 43.40 mg BCE/kg, $p < 0.0001$). Coherently, astaxanthin was detected exclusively in the HP group, with a content of 22.62 mg/kg ($p < 0.0001$). Among the investigated tocopherol isomers, only tocopherol A was detected and expressed as vitamin E. The content of this vitamin did not differ significantly between CTRL and HP groups (21.17 mg/kg vs. 21.82 mg/kg, $p > 0.05$) (Table 6).

Table 6. Main nutritional parameters of egg yolk from laying hens fed CTRL and HP diets. Data are expressed on a fresh weight basis and as mean ± SD.

Nutritional Parameter	CTRL	HP	*p*-Value
Moisture (%)	48.22 ± 0.66	48.64 ± 0.70	0.302
Fat (%)	21.54 ± 0.65	29.23 ± 0.37	<0.0001
Protein (%)	17.57 ± 0.24	16.26 ± 0.19	<0.0001
Total carotenoids (mg/BCE/kg)	19.44 ± 0.88	43.40 ± 0.76	<0.0001
Astaxanthin (mg/kg)	0.00 ± 0.00	22.62 ± 2.27	<0.0001
Vitamin E (mg/kg)	21.17 ± 0.71	21.82 ± 0.32	0.064

Similar to the albumen, the yolk also exhibited a relevant alteration in its element profile due to dietary manipulation (Table 6). Considering the minerals, the quantitative distribution of the minerals differed from that observed in the albumen, as the following quantitative trend emerged: P > Ca > K > Na > Mg. Overall, the contents of these metals ranged from 4452.77–5550.32 mg/kg (P) to 143.33–427.48 mg/kg (Mg) and they were always significantly higher in HP yolks than in the control condition ($p < 0.0001$). Compared to the albumen, a different distribution trend was observed also for the essential trace elements. Indeed, they were found in the order Fe > Zn > Mn > Cu > I > Se and varied from 26.25–64.18 mg/kg to 0.17–0.19 mg/kg. Similar to the minerals, they were found at significantly greater levels in HP samples than CTRL samples (*p*-value between 0.005 and <0.0001). Considering non-essential or potentially toxic elements, a similar profile to that of albumen could be displayed. In fact, Cr, Ni and As were determined at low levels (<0.05 mg/kg), with Ni and As being measured only in HP yolks ($p < 0.0001$). Heavy metals, such as Cd and Pb, were not detected in any of the investigated samples (Table 7).

Table 7. Profile of inorganic elements (mg/kg) in egg yolk from laying hens fed CTRL and HP. Data are expressed on a fresh weight basis as mean ± SD.

Analyte	CTRL	HP	*p*-Value
Na	516.65 ± 2.57	794.98 ± 5.23	<0.0001
K	1039.79 ± 1.18	1879.33 ± 3.71	<0.0001
Mg	143.33 ± 1.38	427.48 ± 3.16	<0.0001
Ca	1396.05 ± 8.54	2002.89 ± 2.83	<0.0001
P	4452.77 ± 17.27	5550.32 ± 4.39	<0.0001
Essential trace elements (mg/kg)			
Mn	8.12 ± 0.02	8.53 ± 0.06	<0.0001
Fe	26.25 ± 0.52	64.18 ± 2.84	<0.0001
Cu	1.66 ± 0.05	1.85 ± 0.03	<0.0001
Zn	18.42 ± 0.41	23.52 ± 1.07	<0.0001
Se	0.17 ± 0.01	0.19 ± 0.01	0.005
I	0.37 ± 0.02	1.08 ± 0.05	<0.0001
Non-essential or potentially toxic elements (mg/kg)			
Cr	0.04 ± 0.01	0.05 ± 0.01	0.010
Ni	0.00 ± 0.00	0.03 ± 0.01	<0.0001
As	0.00 ± 0.00	0.02 ± 0.00	<0.0001
Cd	0.00 ± 0.00	0.00 ± 0.00	-
Pb	0.00 ± 0.00	0.02 ± 0.00	-

Table 8 shows the FA composition determined in the yolk samples under study.

Table 8. Fatty acid (FA) composition (%) and nutritional indices of egg yolk from laying hens fed CTRL and HP diets. Data are expressed on a fresh weight basis and as mean ± SD. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; AI: atherogenicity index; TI: thrombogenicity index; S/P: saturation index; h/H: hypocholesterolemic and hypercholesterolemic FA ratio; PI: peroxidability index.

FA	CTRL	HP	*p*-Value
Myristic acid (C14:0)	0.65 ± 0.06	1.32 ± 0.12	0.001
Pentadecylic acid (C15:0)	0.13 ± 0.04	0.72 ± 0.10	0.001
Palmitic acid (C16:0)	23.12 ± 0.03	20.26 ± 0.06	<0.0001
Stearic acid (C18:0)	10.38 ± 0.16	7.18 ± 0.01	<0.0001
SFA	34.27 ± 0.21	29.47 ± 0.07	<0.0001
Hypogeic acid (C16:1 n-9)	0.78 ± 0.17	0.92 ± 0.06	0.240
Palmitoleic acid (C16:1 n-7)	1.46 ± 0.17	2.64 ± 0.05	0.000
Oleic acid (C18:1 n-9)	33.17 ± 0.04	44.82 ± 0.15	<0.0001
Vaccenic acid (C18:1 n-7)	1.31 ± 0.03	2.81 ± 0.09	<0.0001
MUFA	36.71 ± 0.10	51.19 ± 0.16	<0.0001
Linoleic acid (C18:2 n-6)	22.89 ± 0.08	13.38 ± 0.06	<0.0001
Linolenic acid (C18:3 n-3)	1.14 ± 0.10	0.58 ± 0.08	0.002
Eicosadienoic acid (C20:2 n-6)	0.41 ± 0.05	0.00 ± 0.00	<0.0001
Arachidonic acid (C20:4 n-6)	2.23 ± 0.06	0.80 ± 0.02	<0.0001
Docosahexaenoic acid (C22:6 n-3)	0.25 ± 0.05	1.27 ± 0.06	<0.0001
Docosapentaenoic acid (C22:5 n-6)	1.44 ± 0.15	0.24 ± 0.05	0.000
PUFA	28.36 ± 0.40	16.26 ± 0.03	<0.0001
n-3	1.39 ± 0.12	1.84 ± 0.04	0.007
n-6	25.53 ± 0.09	14.18 ± 0.02	<0.0001
n-6/n-3	18.37 ± 0.76	7.69 ± 0.59	<0.0001
AI	10	0.22 ± 0.00	0.024
TI	0.97 ± 0.22	0.69 ± 0.19	<0.0001
h/H	1.17 ± 0.01	0.87 ± 0.00	<0.0001
PI	28.90 ± 0.29	25.94 ± 0.31	0.002

Dietary supplementation with *H. pluvialis* greatly affected the overall FA composition of egg yolk. Compared to the control condition, egg yolks from hens fed the HP diet were characterized by significantly lower levels of saturated FAs (SFA, 29.47% vs. 34.27%, $p < 0.0001$) and polyunsaturated FAs (PUFA, 16.26% vs. 28.36%, $p < 0.0001$), and significantly higher amounts of monounsaturated FAs (MUFA, 51.19% vs. 36.71%, $p < 0.0001$). These differences were determined by certain predominant FAs that displayed a significant quantitative variation between the study groups. Specifically, palmitic acid (CTRL group: 23.12%, HP group: 20.26%, $p < 0.0001$), oleic acid (CTRL group: 33.17%, HP group: 44.82%, $p < 0.0001$) and linoleic acid (CTRL group: 22.89%, HP group: 13.38%, $p < 0.0001$) were the most abundant FAs in the SFA, MUFA, and PUFA categories, respectively. However, other nutritionally relevant FAs that positively varied as a result of the manipulation of the hens' diet were palmitoleic acid ($p < 0.05$), vaccenic acid ($p < 0.0001$), docosahexanoic acid ($p < 0.0001$) and docosapentaenoic acid ($p < 0.0001$). Given these differences in the FA composition of CTRL and HP yolks, significant changes were also observed in the total n-3 FAs, n-6 FAs and n-6/n-3 ratio. Specifically, a significant increase in n-3 FAs (1.39 vs. 1.84, $p < 0.05$) and a significant decrease in n-6 FAs (25.53 vs. 14.18, $p < 0.0001$) were observed in HP yolks, when compared with the CTRL counterpart. Consequently, the n-6/n-3 ratio significantly decreased in HP yolk, becoming more balanced from a nutritional point of view (18.37 vs. 7.69, $p < 0.0001$).

The atherogenic index (AI) and thrombogenic index (TI) were lower in the HP group ($p < 0.05$ and $p < 0.0001$, respectively), whereas the hypocholesterolemic/hypercholesterolemic ratio (HH) was reduced ($p < 0.0001$). The peroxidability index (PI) was significantly lower in HP compared to CTRL ($p < 0.01$).

3.4. Sensory Analysis

3.4.1. E-Eye

Principal component analysis (PCA) of E-Eye data (Figure 1) revealed a clear separation between CTRL and HP samples. The discrimination index was 85. PC1 explained 75.67% of the total variance, while PC2 accounted for 18.72%, for a cumulative variance exceeding 94%. CTRL samples were associated with lighter color tones, whereas HP samples were associated with darker and more intense color codes in the red–orange range. The HP group showed greater dispersion compared to CTRL.

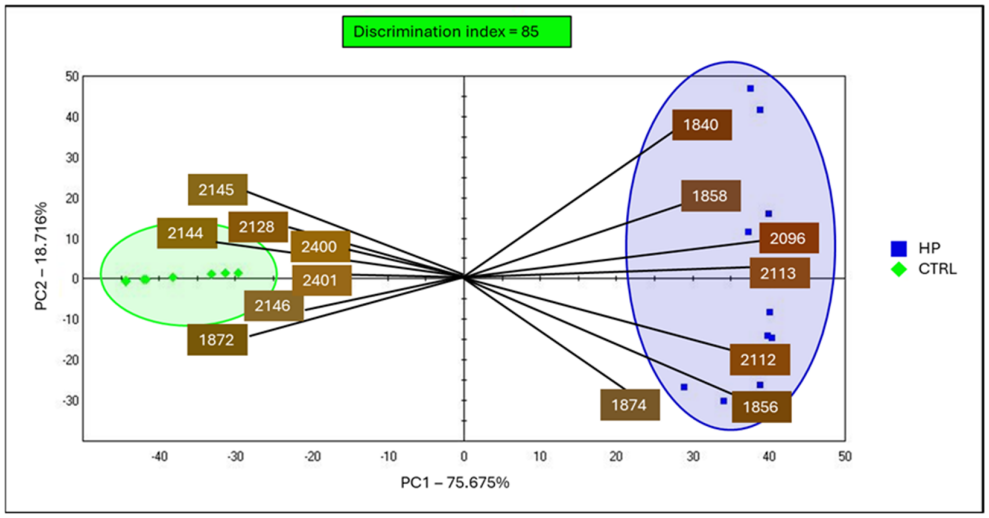

Figure 1. Principal component analysis (PCA) score and loading plot of E-eye data based on RGB color parameters of egg yolks from the control (CTRL) and *Haematococcus pluvialis* (HP) groups. PC1 and PC2 explain 75.67% and 18.72% of the total variance, respectively. CTRL samples (green diamonds) are clustered on the negative side of PC1 and are associated with lighter color tones, whereas HP samples (blue squares) are distributed on the positive side of PC1 and are associated with darker and more intense red–orange color codes. Ellipses represent group dispersion. The discrimination index was 85, indicating a clear separation between groups.

3.4.2. E-Nose

PCA of E-Nose data (Figure 2) showed that PC1 explained 60.12% of the total variance, while PC2 accounted for 23.91%, for a cumulative variance of approximately 84%. The score plot showed partial separation between CTRL and HP samples, with overlap between groups. HP samples exhibited greater dispersion compared to CTRL. The discrimination index was −13. Loadings analysis indicated that CTRL samples were mainly associated with sensors P30/2, P30/1, P10/1, and T70/2, whereas HP samples were associated with sensors LY2/G, LY2/GH, LY2/AA, and LY2/gCTL.

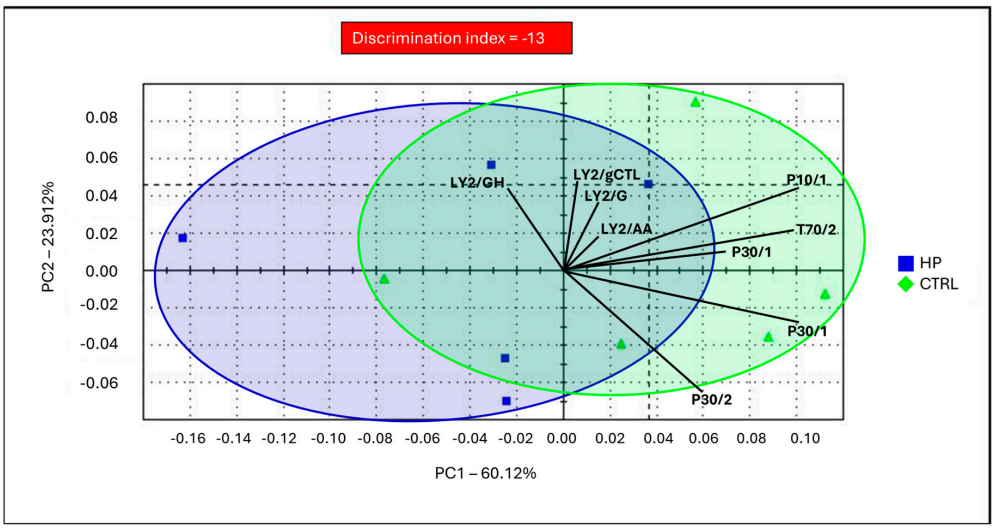

Figure 2. Principal component analysis (PCA) score and loading plot of E-nose data obtained from egg samples of the control (CTRL) and *Haematococcus pluvialis* (HP) groups. PC1 and PC2 explain 60.12% and 23.91% of the total variance, respectively. CTRL samples (red symbols) and HP samples (blue symbols) show partial separation with overlapping regions, indicating limited discrimination between groups. Ellipse areas represent sample distribution within each group. The discrimination index was −13. Loading vectors indicate the contribution of individual sensors to sample distribution along the principal components.

4. Discussion

The present study demonstrates that dietary supplementation with dried *Haematococcus pluvialis* biomass can effectively enhance the nutritional and functional quality of eggs without negatively affecting their main physical characteristics. The observed effects were particularly evident in yolk pigmentation, carotenoid deposition, mineral enrichment, lipid profile modulation, and instrumental sensory traits.

Dietary inclusion of *H. pluvialis* did not substantially affect most external and internal physical egg parameters, confirming that microalgae supplementation generally does not interfere with egg formation processes. This finding is consistent with previous studies reporting no significant changes in egg weight, albumen quality, or shell traits following the inclusion of microalgae or carotenoid sources in laying hen diets [39–41]. The limited impact on physical traits suggests that the physiological mechanisms regulating egg formation remain stable despite the introduction of functional feed ingredients.

In the present study, hens fed the *H. pluvialis* diet showed a reduction in oviposition rate and egg mass, while body weight and egg weight were not affected. This pattern suggests that dietary treatment influenced laying frequency rather than the formation of individual eggs. These findings are not fully consistent with previous studies, where astaxanthin or microalgae supplementation generally did not negatively affect productive performance. Considering the very low inclusion level used in the present study, the biological mechanism underlying this effect remains unclear. Therefore, these results should be interpreted with caution and require further investigation. Although a reduction in oviposition rate was observed in the HP group, the marked improvements in yolk pigmentation, carotenoid enrichment, mineral composition, fatty acid profile, and nutritional quality indices suggest that dried *H. pluvialis* biomass may represent a valuable strategy for the production of premium functional eggs. However, further studies are needed to evaluate the economic balance between productive performance and product added value under commercial production conditions.

In contrast, yolk color was markedly enhanced in the supplemented group, reaching the highest values on the DSM color scale. This effect is directly attributable to the high content of astaxanthin in *H. pluvialis*, which is efficiently absorbed in the intestine and transported to the ovary via lipoproteins. The deposition of carotenoids in the yolk follows well-established pathways involving micellar solubilization, intestinal uptake, hepatic metabolism, and subsequent incorporation into very low-density lipoproteins (VLDLy), which are selectively taken up by the developing oocyte [42]. Compared to other carotenoids, astaxanthin exhibits a higher pigmentation efficiency, resulting in more intense orange–red coloration [22]. From a market perspective, darker yolks are often associated with higher perceived quality and consumer preference, suggesting a potential added value of eggs obtained from hens fed microalgae-based diets.

The increase in total carotenoids and the exclusive presence of astaxanthin in the yolk of the supplemented group confirm the effectiveness of *H. pluvialis* as a natural source of bioactive compounds for egg biofortification. Beyond their coloring properties, carotenoids exert strong antioxidant activity, contributing to the protection of lipids from oxidative degradation and potentially improving the shelf life and nutritional value of eggs [43]. In addition, the specific stereochemistry and esterified forms of astaxanthin enhance its bioavailability and biological activity [44], further supporting its relevance as a functional dietary component.

A notable outcome of the present study is the marked enrichment of mineral elements in both albumen and yolk. The increase in essential minerals such as Ca, Mg, K, Fe, Zn, and I suggests that *H. pluvialis* biomass represents an effective vector for micronutrient transfer from feed to eggs. Microalgae are known to contain highly bioavailable minerals, which can be efficiently absorbed and incorporated into animal tissues [45]. The enrichment of eggs with essential micronutrients enhances their nutritional value and supports their role as functional foods, contributing to human dietary requirements related to metabolic regulation, immune function, and antioxidant defense.

The fatty acid profile of the yolk was significantly modulated by dietary treatment, with a reduction in saturated fatty acids and a marked increase in monounsaturated fatty acids. The increase in oleic acid (C18:1 cis) may be related to the lipid composition of *H. pluvialis*, where astaxanthin is predominantly present in esterified forms associated with C18 fatty acids [46–48]. The observed decrease in polyunsaturated fatty acids, particularly n-6 fatty acids, resulted in a significant decrease in the n-6/n-3 ratio, indicating a more balanced fatty acid profile. These changes were reflected in improved nutritional indices, including lower atherogenic and thrombogenic indices and hypocholesterolemic/hypercholesterolemic ratio, indicating a more favorable lipid profile. In the present study, the AI was significantly higher in the CTRL group than in the HP group. This index reflects the relative contribution of the fatty acid profile to atherogenic potential, with lower values generally considered more favorable from a nutritional perspective. Therefore, the reduction observed in eggs from hens fed diets supplemented with *H. pluvialis* suggests an improvement in the nutritional quality of the lipid fraction compared with the control group [49]. A similar trend was observed for the thrombogenic index (TI), which was significantly lower in the HP group than in the CTRL group. The TI estimates the propensity of the fatty acid profile to promote thrombus formation, and lower values are generally associated with a more favorable lipid composition [50]. Although AI and TI are widely used to assess the nutritional quality of dietary lipids, these indices should be interpreted with caution because they do not fully account for the complexity of the fatty acid profile, total fat content, food matrix effects, or actual dietary intake. Therefore, they should be regarded as complementary indicators that provide additional information on lipid quality rather than definitive predictors of the health effects of food consumption [51,52]. Moreover,

the reduction in the peroxidability index suggests improved oxidative stability of the yolk lipids, likely due to the combined effect of reduced PUFA content and increased antioxidant availability. Instrumental sensory analysis provided further insights into the effects of dietary supplementation.

The E-eye analysis clearly discriminated between groups, confirming the strong impact of carotenoid deposition on yolk color. In contrast, the E-nose analysis showed only partial separation between groups, indicating that volatile profiles were less affected by dietary treatment. This result can be explained by the relationship between lipid composition and volatile compound formation. Eggs from the control group, characterized by higher PUFA levels, may be more prone to oxidative processes leading to the formation of aldehydes, ketones, and hydrocarbons, which are key contributors to aroma [53].

Conversely, the presence of astaxanthin and the higher MUFA content in the supplemented group may have contributed to greater oxidative stability, limiting the formation of volatile compounds and resulting in a less defined aromatic differentiation. These findings highlight the complementary role of artificial sensory systems in detecting both evident and subtle changes in food quality.

Despite the promising results, some limitations should be acknowledged. The study was conducted during the early laying phase; therefore, the eggs evaluated may not fully correspond to standard commercial market classes, particularly medium and large eggs produced during peak laying. This experimental timing was selected to assess the early transfer and deposition of bioactive compounds from dietary *H. pluvialis* biomass into eggs during a phase of active physiological adaptation. However, further studies are needed to confirm these findings during peak production and on eggs belonging to standard commercial size classes under commercial farming conditions. The study was conducted under controlled experimental conditions and over a relatively short period; therefore, further research is needed to evaluate long-term effects and scalability under commercial farming systems. In addition, the influence of different inclusion levels of *H. pluvialis* and potential interactions with other dietary components should be further investigated.

Notably, the present study provides a comprehensive multilevel characterization of egg quality, integrating compositional, nutritional, and instrumental sensory analyses. This integrated approach represents a novel contribution to the evaluation of functional feed ingredients in poultry nutrition and supports the development of innovative strategies for the production of value-added eggs.

5. Conclusions

The results of the present study demonstrate that dietary supplementation with dried *Haematococcus pluvialis* biomass can enhance egg quality through natural biofortification. While most physical parameters were not affected, significant improvements were observed in yolk pigmentation, carotenoid content, mineral composition, and lipid profile, leading to eggs with increased nutritional and functional value. In particular, the enrichment of yolk with astaxanthin and essential micronutrients, together with the improvement in lipid-related nutritional indices, highlights the potential of *H. pluvialis* as a sustainable feed ingredient in poultry production. Instrumental sensory analysis further confirmed the impact of dietary treatment on visual attributes, while indicating more limited effects on volatile profiles. However, a reduction in oviposition rate and egg mass was observed, suggesting that the effects of supplementation should be evaluated considering both quality enhancement and productive performance. Overall, the use of *H. pluvialis* biomass may represent a promising approach for the development of value-added eggs with enhanced nutritional properties, supporting innovative and sustainable feeding strategies in the

poultry sector. Further research is warranted to evaluate long-term effects and optimize inclusion levels under commercial farming conditions.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Messina, Department of Veterinary Sciences (protocol code 24/2025, approved on 30 March 2025).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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