Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: D. Lanzoni, M. Skřivan, M. Englmaierová, E. Petrosillo, L. Marchetti, V. Skřivanová, V. Bontempo, R. Rebucci, A. Baldi & C. Giromini (2025) Effects of dietary hemp co-product inclusion on laying hens performances and on egg nutritional and functional profile, Italian Journal of Animal Science, 24:1, 248-265, DOI: 10.1080/1828051X.2024.2449445

To link to this article: https://doi.org/10.1080/1828051X.2024.2449445

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Published online: 19 Jan 2025.

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ITALIAN JOURNAL OF ANIMAL SCIENCE
2025, VOL. 24, NO. 1, 248–265
https://doi.org/10.1080/1828051X.2024.2449445

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RESEARCH ARTICLE

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Effects of dietary hemp co-product inclusion on laying hens performances and on egg nutritional and functional profile

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ABSTRACT

The European Union promotes a circular economy by valorising food industry scraps as co-products for animal feed. Hemp (*Cannabis sativa* L.) co-products are promising for their nutritional and functional profile, but their use in animal nutrition remains limited. This study evaluated the effect of increasing levels (0%, 3%, 6%, 9%) of a hemp co-product (variety Futura) from hempseed cleaning, included in laying hens' diets (control, T_3, T_6, T_9), on performance and the nutritional/functional quality of egg yolks, focusing on total phenolic content (TPC) and antioxidant activity (FRAP and ABTS assays), also assessed after green chemical extraction and *in vitro* digestion. Including up to 9% hemp co-product in diets did not affect dry matter and protein digestibility but improved the functional profile. A 9% inclusion significantly increased egg mass production ($p < 0.01$) and reduced saturated fatty acid content (T_9: 7672 mg/100 g; T_6: 8534 mg/100 g; T_3: 8837 mg/100 g; control: 8742 mg/100 g), alongside an improved n-6/n-3 fatty acid ratio. Cholesterol levels were significantly lower than the control in all concentrations tested, with better results for T9 ($p < 0.01$). Antioxidant activity, measured *via* ABTS after *in vitro* digestion, increased significantly (control: 601.12 ± 42.40 mg TE/100 g; T_3: 773.17 ± 43.77 mg TE/100 g; T_6: 765.64 ± 38.71 mg TE/100 g; T_9: 843.48 ± 38.71 mg TE/100 g; $p < 0.05$), despite no differences in TPC. In conclusion, the inclusion of 9% hemp co-product in laying hens' diets improved egg production and yolk quality, highlighting its potential for animal feed. However, the composition variability requires further evaluation to ensure future large-scale application.

HIGHLIGHTS

- Farm to Fork strategy aims to guide the agri-sector to sustainable food system.
- Hemp co-products are featured by an interesting nutritional and functional profile.
- The 9% inclusion of the hemp co-product improved the performance of laying hens and the nutritional and functional profile of eggs.

ARTICLE HISTORY
Received 9 September 2024
Revised 30 November 2024
Accepted 30 December 2024

KEYWORDS
Antioxidant; digestion; hemp; poultry; yolks

Introduction

In recent decades, the European Union has been actively working to ensure a clean and circular economy (Garcia et al. 2020; Beltran et al. 2022). In this panorama, the Farm to Fork strategy plays an important role as it aims to guide the agricultural sector towards healthier and more environmentally friendly food systems, making them more sustainable than the current ones (European Union 2020).

To achieve this, one possible strategy is to use the large amount of non-edible biomass produced along the food chain and to valorise it as co-product for the livestock sector (FAO 2011; Rakita et al. 2021). To date, scientific research is deepening the use of co-products derived from hemp (*Cannabis sativa L.*) (Bailoni et al. 2021; Vastolo et al. 2021; Lanzoni et al. 2023a). Hemp is well-known in the food/feed sector for the important nutritional (25–35% lipid content, 20–25% protein rich in essential amino acids and 20–30%

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/1828051X.2024.2449445.

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carbohydrates, a major part of which is dietary fibre) and functional profile (presence of phenols and anti-oxidant molecules) of its seeds (Farinon et al. 2020; Lanzoni et al. 2023b).

Despite this, the use of hemp co-products is still in an early stage. As demonstrated in literature, hemp co-products can be summarised as: whole plant, stems, chaff, stalks, hulls, HSs cakes, leaves and flower (before and after cannabinoid extraction) (Vastolo et al. 2021; Ely and Fike 2022). As shown by Ely and Fike (2022) and Vastolo et al. (2021), hemp co-products are characterised by an interesting nutritional and functional profile, which unfortunately varies widely due to many factors such as environmental, agro-ecological conditions, genotype, harvesting techniques and the transformation processes. To date, through Regulation 2022/1104 of 1 July 2022, the European Union only authorised HSs, HSs oil, HSs cake, hemp flour and hemp fibre (both originated by stems) for animal feeding, as reported in the European Catalogue of Feed Materials (European Union 2022).

For this reason, it is important to investigate the use of new hemp-based matrices, which are able to guarantee high animal performances and improve the nutritional aspect of animal products. The starting point for future research is related to the fact that important results have been obtained with the incorporation of HSs in the livestock industry, particularly in the laying hen sector. Indeed, HSs are able not only to increase the performance of the treated animal, but at the same time to improve the nutritional and functional profile of the eggs (Gakhar et al. 2012; Goldberg et al. 2012; Neijat et al. 2014; Shahid et al. 2015; Neijat et al. 2016; Konca et al. 2019; Mierliță 2019; Skřivan et al. 2019).

In the light of the above, it is possible to assume that such important results can also be achieved through the enhancement of hemp co-products. For this reason, the aim of this work was to evaluate how different levels of inclusion (3%, 6% and 9%) of a hemp co-product, deriving from the cleaning of the whole HSs (leaves, non-standard H.S., hulls and stems), in the diet of laying hens are able to modulate the production performance and nutritional/functional profile of egg yolks. Specifically, in addition to assessing the fatty acid profile and cholesterol content, the presence of bioactive compounds such as tocopherol, retinol, β-carotene, lutein and zeaxanthin were analysed. Finally, the total phenolic content (TPC) and anti-oxidant profile of egg yolks were evaluated following green chemical extraction and *in vitro* digestion.

Material and methods

Material

The hemp co-product (*C. sativa* L, variety Futura) (BIOHEMP, Prague, Czech Republic), harvested at the end of September 2022, was generated during the cleaning of whole HSs using sieves and airflow. Hemp co-product was characterised by the presence of leaves, non-standard HSs, HSs hulls and stems. The resulting sample was stored in the dark at room temperature.

Experimental design

240 hens (Bovans Brown) aged 21 weeks took part in the experiment. The trial lasted nine weeks (two weeks of adaptation and seven of experiment), which were comprehensive of the peak production phase, as from week 30, production gradually decreases (Jin et al. 2010; Gonzalez-Uarquin et al. 2021). The hens were divided into four dietary treatments [control, T_3, T_6 and T_9) (selected on the basis of our previous work; Skřivan et al. 2019) with increasing inclusion of the hemp co-product in the diet (0%, 3%, 6% and 9%, respectively), as shown in Table 1. All diets, as reported in Table 2, were formulated to contain similar levels of metabolisable energy and protein content, therefore, following increasing concentrations of the hemp co-product featured by contained energy levels, vegetal oil (rapeseed oil) was gradually increased to maintain isoenergetic diets among groups.

Table 1. Composition of the dietary treatments fed to laying hens (control, T3, T6 and T9).

Ingredient (%)	Control 0%	T_3 3%	T_6 6%	T_9 9%
Wheat (11% CP)	39.10	37.70	36.20	34.10
Corn	19.60	19.60	19.60	19.60
Soybean meal (44%)	24.40	24.10	23.70	23.70
Wheat bran	4.10	2.70	1.30	0.00
Hemp co-product	0.00	3.00	6.00	9.00
Rapeseed oil	1.97	2.34	2.79	3.48
CaH_2PO_4	0.78	0.75	0.72	0.69
Sodium bicarbonate	0.30	0.30	0.30	0.30
Sodium chloride	0.20	0.20	0.20	0.20
Limestone-grit (1–2 mm)	8.90	8.60	8.40	8.10
L-Lysine hydrochloride	0.00	0.02	0.06	0.07
DL-Methionine	0.14	0.16	0.18	0.20
L-Threonine	0.01	0.03	0.05	0.06
AMINOVITAN[a]	0.50	0.50	0.50	0.50

Values are reported in %.
[a]AMINOVITAN (in 1 kg): vitamin A 8.721 IU; vitamin D3 3.000 IU, niacin 25 mg; calcium pantothenate 8 mg; thiamine 2 mg; riboflavin 5 mg, pyridoxin 4 mg; folic acid 0.5 mg; biotin 0.075 mg; cobalamin 0.01 mg; choline chloride 250 mg; menadione 2 mg; betaine 100 mg; butylated hydroxytoluene 7.5 mg; ethoxyquin 5.6 mg; butylhydroxyanisole 1 mg; DL-methionine 0.7 g; manganese 70 mg; zinc 50 mg; iron 40 mg; copper 6 mg; iodine 1 mg; cobalt 0.3 mg; selenium 0.2 mg.
T: treatments; CP: crude protein.

Table 2. Chemical composition of dietary treatments fed to laying hens (control, T3, T6, T9).

Nutrient content	Control 0%	T_3 3%	T_6 6%	T_9 9%	HCP
Dry matter (g/kg)	899	901	902	902	889
Ashes (g/kg)	124	142.8	128.9	132.7	148.3
Crude protein (g/kg)	173	174	170	175	156
Ether extract (g/kg)	36.9	37.7	39.1	40.7	64.8
NDF (g/kg)	190	207	208	193	423
ADF (g/kg)	33.7	39.1	46.7	53.7	281
ADL (g/kg)	7.2	8.1	11.9	13.4	107.1
ME_N (MJ/kg)	10.9	11.0	10.9	10.9	5.2
Calcium (g/kg)	36.2	36.0	36.3	36.2	32.2
Phosphorus (g/kg)	5.69	5.73	5.65	5.74	5.25
α-Tocopherol (mg/kg)	40.1	42.0	44.0	47.3	19.2
γ-Tocopherol (mg/kg)	14.1	16.9	17.4	18.5	12.0
Retinol (mg/kg)	1.89	2.10	2.40	3.08	–
Lutein (mg/kg)	2.46	3.36	4.32	5.54	26.4
Zeaxanthin (mg/kg)	2.21	1.92	1.62	1.57	1.02
β-Carotene (mg/kg)	0.267	0.689	1.173	1.071	4.90

HCP: Hemp co-product; MEN: Metabolisable energy; T: treatment; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; ME: metabolisable energy.

More precisely, the hens were housed in three-floor enriched cages and divided into ten hens/cages for a total of six cages per group ensuring optimal microclimatic and technological conditions for the considered genotype, with a daily light cycle length of 16 h (02.30–18.30). The cages had a surface area of 7.560 cm^2. A nest box, feeder (12 cm per hen) and three nipple water dispensers were included in each cage. Additionally, the cages were equipped with a perch (15 cm per hen), a dust bath and equipment for claw abrasion. Room temperature was maintained at 20 – 22 °C, and the light intensity was approximately 10 lx in the central storey.

At the beginning and end of the experiment all animals were weighed. The health status and mortality rate were assessed daily, while the feed intake per cage and group was recorded weekly.

The number of eggs laid was registered daily.

Methods

Chemical analysis of co-product and diets

The chemical analysis of the hemp co-product and diets was carried out following the official methods reported by AOAC (2005). In particular, DM was obtained by drying the sample in a forced-air oven at 65 °C for 24 h (AOAC method 942.05), while ashes by incinerating the samples in a muffle at 550 °C for 3 h (AOAC method 942.05). In parallel, CP content was measured by the Kjeldahl method (AOAC method 2001.11) and the ether extract by extraction in petroleum ether, using a Tecator 1045 Soxtec Extraction Unit (DM 21/12/1998). Finally, the fibrous fractions were detected using the protocol reported by Van Soest et al. (1991). Calcium content was measured in hydrochloric acid by atomic absorption spectrometry using the ContrAA 700 F instrument (Analytik Jena AG, Jena, Germany), as described by Skřivan et al. (2019), while phosphorus content was determined using the vanadate-molybdate reagent, as reported by AOAC (2005; method 956.17).

The fatty acid profile [saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs)] were classified following chloroform and methanol extraction of the total lipid fraction as shown by Folch et al. (1957).

Subsequently, alkaline trans-methylation of fatty acids was performed as reported by Raes et al. (2003). More specifically, the fatty acid methyl esters (FAMEs) were determined by gas chromatography (HP chromatograph 6890, Agilent Technologies, Inc.) with a 60 m DB-23 capillary column (150–230 °C) and a flame ionisation detector, using specific temperature programmes, as featured in the Supplementary Materials. Finally, the fatty acids were identified by their retention times compared with standards (PUFA 1, PUFA 2, PUFA 3 and a 37–component FAME mixes (Supelco, Bellefonte, PA, USA)).

The concentration of α-tocopherol, γ-tocopherol, β-carotene and retinol was identified following saponification and extraction in diethyl ether in accordance with European standards (European Committee for Standardisation 2000). Lutein and zeaxanthin contents were calculated using HPLC equipped with a diode-array detector (VP series; Shimadzu, Kyoto, Japan) as reported by Skřivan et al. (2019) and Froescheis et al. (2000). A wavelength of 450 nm was applied for the detection. More specifically, a Kinetex C18 column (100 × 4.6 mm; 2.6 um (Phenomenex, Torrance, USA) was used. After preparing the eluents [(A): acetonitrile:water:ethyl acetate (88:10:2) and (B): acetonitrile:water:ethyl acetate (88:0:15)], the samples were analysed by comparing with appropriate calibration standards (Sigma-Aldrich, Prague, Czech Republic). The analysis for the identification of lutein and zeaxanthin was of 18 min.

In vitro digestion and digestibility of hemp co-product and diets

The hemp co-product and diets were digested following Regmi et al. (2009) with minor modifications (Lanzoni et al. 2023b), as reported in Supplementary Materials.

At the end of digestion, the samples were vacuum filtered through paper filters (Whatman 54 Florham Park, NJ), collecting the undigested fraction (UF). UF

was then incubated overnight at 50 °C and used to determine the DM digestibility.

$$\text{Digestibility (\% DM)} = (\text{sample DM} - \text{UF DM})/\text{sample DM} \times 100)$$

For diets only, filters were used to assess protein digestibility, using the Kjeldahl method (AOAC 2005).

In parallel, aliquots (around 2 mL) were taken every hour of digestion for the hemp co-product, while for the diets at the end of digestive process, to monitor TPC and antioxidant activity, as described below.

Determination of physical parameters of eggs

The physical parameters of the eggs were determined at the end of the trial.

A whole day of egg production was analysed and the values were averaged per cage ($n = 6$). More precisely, following the measurement of the total weight and surface, the eggs were broken and the individual fractions were isolated. For each component, the weight was determined and based on this, the percentages of white, yolk and shell were subsequently identified.

The shell's breaking strength was determined in the vertical axis using an Instron 3360 apparatus (Instron, Norwood, MA, USA). After removal of the shell membranes, the thickness was measured using a micrometer.

Finally, the yolk colour was analysed using DSM yolk colour fan (DSM Nutritional Products, Basel, Switzerland) and Minolta CR-300 colorimeter (Konica Minolta, Osaka, Japan) (Skřivan et al. 2019).

Nutritional and functional profile of egg yolks

Eggs for determination of fatty acid, cholesterol, vitamin and carotenoid content in the yolks were collected at the end of the trial (3 yolks (= 1 sample) from each cage, $n = 6$).

The content and lipid profile were determined as previously reported (Folch et al. 1957; Raes et al. 2003) by gas chromatography (HP chromatograph 6890, Agilent Technologies, Inc). The fatty acids were identified by their retention times compared with standards. PUFA 1, PUFA 2, PUFA 3 and 37–component FAME mixes (Supelco, Bellefonte, PA, USA) were used as standards.

In parallel, the atherogenic and thrombogenic indices were quantified as described by Ulbricht and Southgate (1991) and the peroxidation index in accordance with Cortinas et al. (2003). The α-tocopherol, γ-tocopherol, β-carotene, retinol, lutein and zeaxanthin present in egg yolks were analysed as indicated above (European Committee for Standardisation 2000; Froescheis et al. 2000; Skřivan et al. 2019), using HPLC (VP series; Shimadzu, Kyoto, Japan) equipped with a diode array detector. The yolks for vitamin determination were subjected to alkaline saponification with 60% potassium hydroxide followed by the appropriate extraction with diethyl ether.

Finally, the cholesterol content was measured as reported by Skřivan et al. (2019). Briefly, following saponification of the lipid component, the non-saponified fraction was subjected to extraction in ethyl ether. The derivatives thus obtained were quantified using a gas chromatograph equipped with a SAC-5 capillary column (Supelco, Bellefonte, USA) in isothermal conditions with a temperature of 285 °C.

Green chemical extraction and in vitro digestion of egg yolks

Before the extraction and digestion process, the yolks of each individual egg were subjected to the freeze-drying process (3 yolks (=1 sample) from each cage, $n = 6$). After this, pools were created for each dietary treatment (control, T_3, T_6, T_9). More precisely, the biphasic green chemical extraction (50:50; H_2O:EtOH) was performed as described in supplementary materials, following the protocol of Brighenti et al. (2017) with minor modifications as reported by Lanzoni et al. (2024).

In vitro digestion was performed following the Infogest protocol (Minekus et al. 2014; Brodkorb et al. 2019) (Supplementary materials). At the end of digestion, the samples were used for the evaluation of TPC and antioxidant activity with the Ferric Reducing Antioxidant Power (FRAP) and 2,2′-azinobis-(3–ehtylbenzothiazoline-6–sulfonic acid (ABTS) assays.

Total phenolic content and antioxidant activity of hemp co-product, diets and eggs

Total phenolic content was assessed with the Folin-Ciocalteu assay, as reported by Attard (2013).

In parallel, antioxidant activity was evaluated by FRAP and ABTS method, according to the protocols of Abdelaleem and Elbassiony (2021) and Re et al. (1999), respectively, with minor modifications as reported by Lanzoni et al. (2024). The results were expressed as mg tannic acid equivalent (TAE)/100 g, mg $FeSO_4$/100 g and mg Trolox equivalent (TE)/100 g, for TPC, FRAP and ABTS respectively. The methodology is detailed in the Supplementary materials.

Statistical analysis

Data on animal performances, physical characteristics of eggs, egg yolks lipid profile, cholesterol content and antioxidant molecules were analysed using one-way Anova with GLM procedure in SAS software (Version 9.3; 2003). Differences between the groups were tested by the Duncan test. The main effect was the dose of hemp co-product. The mathematical statistical model was as follows:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where Y_{ij} was the value of trait; μ was the overall mean; α_i was the effect of hemp co-product inclusion ($i = 0\%$, 3%, 6%, 9%) and eij was the random residual error.

The cage was the experimental unit ($n = 6$).

At the same time, DM/protein digestibility, TPC, ABTS and FRAP data of hemp co-product, diets and egg yolks were analysed by one-way Anova followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are reported as mean ± SEM.

Values are considered statistically significant for a 95% confidence interval (p-value = 0.05).

Results

Nutritional and functional profile of in vitro digested hemp co-product and diets

As shown in Table 2, the hemp co-product had an interesting nutritional profile, mainly due to its high protein content (156 g/kg). At the same time, it presented a discrete lipid level (64.8 g/kg), enriched by the high presence of PUFAs (1225.61 mg/kg) compared to MUFAs (263.47 mg/kg) and SFAs (430.61 mg/kg), which consequently improved the lipid profile of the diets, as reported in Table 3. In parallel, it showed a high content of ashes (148.3 g/kg) and fibre (423 g/kg, 281 g/kg and 107.1 g/kg, respectively for NDF, ADF and ADL), causing a reduced DM digestibility (36.09 ± 1.29%).

Despite this, increasing inclusions of hemp co-product in the diet (control, 3%, 6% and 9%) did not result in statistically significant differences in DM (64.21 ± 0.97%; 64.06 ± 1.39%; 63.85 ± 1.16%; 62.14 ± 1.40%) and protein (81.13 ± 6.62%; 79.10 ± 2.60%; 77.71 ± 7.06%; 78.82 ± 3.43%) digestibility, respectively, for control, T_3 (3%), T_6 (6%) and T_9 (9%).

As previously reported, aliquots of the hemp co-product were collected every hour during the digestion process to investigate the behaviour of phenolic (Figure 1a) and antioxidant compounds (Figure 1b, c).

The hemp co-product presented an increasing trend in TPC (Figure 1a) from the beginning of the digestive process (1072.66 ± 115.82 mg TAE/100 g), peaking at the end of the gastric phase (1752.16 ± 66.73 mg TAE/100 g) ($p < 0.05$). At the start of the intestinal incubation (1127.37 ± 40.17 mg TAE/100 g), a statistically significant ($p < 0.05$) momentary decrease was observed, although it declined at the end of the digestive process, resulting in a value highly comparable to that previously registered at the end of the gastric phase (1507.50 ± 9.72 mg TAE/100 g).

The same trend was also obtained for the FRAP assay (Figure 1b), confirming the peak at the end of gastric digestion (169.80 ± 14.84 mg $FeSO_4$/100 g), significantly higher ($p < 0.05$) than at any other timepoint.

In contrast, the ABTS method (Figure 1c) reported an opposite course. More specifically, the beginning of the gastric phase (969.07 ± 56.51 mg TE/100 g) showed a significant ($p < 0.05$) decrease in antioxidant activity compared to the beginning of digestion (2852.03 ± 183.27 mg TE/100 g). Although no appreciable differences were observed during the gastric incubation, the start of the intestinal phase showed a marked ($p < 0.05$) increase (4573.77 ± 39.38 mg TE/100 g), which remained constant over the next three hours, before declining in the last hour of incubation (3782.09 ± 223.58 mg TE/100 g) ($p < 0.05$).

As presented in Figure 1d, increasing inclusions of the hemp co-product improved the TPC of T_3 (3%), T_6 (6%) and T_9 (9%) compared to the control (0%). More precisely, T_3 showed a significantly ($p < 0.05$) higher phenolic content (755.53 ± 29.07 mg TAE/100 g) than the control (670.42 ± 3.88 mg TAE/100 g) and highly similar to T_6 (773.68 ± 7.37 mg TAE/100 g). Better results were obtained for T_9 (841.89 ± 29.96 mg TAE/100 g) although comparable with T_6. Similar trends were observed for the FRAP assay (Figure 1e), with statistically higher values ($p < 0.05$) for T_9 (46.48 ± 2.48 mg $FeSO_4$/100 g) compared to the control (27.89 ± 2.01 mg $FeSO_4$/100 g), T_3 (31.99 ± 2.29 mg $FeSO_4$/100 g) and T_6 (36.23 ± 3.42 mg $FeSO_4$/100 g).

In parallel, the antioxidant activity assayed by the ABTS method (Figure 1f) confirmed better results for the 9% (3451.51 ± 143.44 mg TE/100 g) inclusion of the co-product in the diet, significantly higher ($p < 0.05$) than the control (2963.76 ± 62.04 mg TE/100 g) and comparable with the inclusion of 3% and 6% (3125.50 ± 78.73 mg TE/100 g; 3221.29 ± 129.15 mg TE/100 g, respectively). However, although an increasing trend was observed from control to T_6, no statistically significant difference was observed between any of the diets considered.

Table 3. Fatty acid profile of hemp co-product and dietary treatments.

	Control 0%	T_3 3%	T_6 6%	T_9 9%	HCP
Caproic acid (C6:0)	0.056	0.07	0.10	0.11	
Caprylic acid (C8:0)	0.21	0.21	0.23	0.24	
Capric acid (C10:0)	0.42	0.43	0.45	0.50	
Lauric acid (C12:0)	1.87	2.76	3.18	3.41	1.55
Trydecylic acid (C13:0)	0.08	0.12	0.13	0.15	
Myristic acid (C14:0)	3.35	7.14	9.10	10.29	42.69
Myristoleic acid (C14:1-n5)	0.22	0.44	0.56	0.80	4.18
Pentadecylic acid (C15:0)	1.82	1.82	2.18	2.20	1.76
Palmitic acid (C16:0)	388.02	381.15	450.82	449.34	254.65
Palmitoleic acid (C16:1, n-7)	7.28	7.99	9.92	11.88	10.32
Margaric acid (C17:0)	3.12	3.20	3.97	4.07	3.20
Stearic acid (C18:0)	84.56	89.45	119.03	123.52	94.57
Olenic acid (C18:1, n-9)	1318.03	1409.32	1853.58	2126.27	214.59
Vaccenic acid (C18:1, n-7)	66.07	71.56	92.42	112.32	26.02
Linoleic acid (C18:2, n-6)	1479.78	1490.07	1687.67	1729.22	856.32
Conjugated Linoleic Acid (CLA; 18:2 c9,t11)	0.61	0.64	0.73	0.83	15.46
Conjugated Linoleic Acid (CLA; 18:2 t10,c12)	0.21	0.23	0.34	0.41	0.22
γ-linoleic acid (C18:3, n-6)	3.11	3.40	7.96	9.25	43.02
α-linolenic acid (C18:3, n-3)	241.35	256.47	334.91	381.73	290.06
Arachidic acid (C20:0)	14.89	19.05	24.86	29.76	31.40
Gondoic acid (C 20:1, n-9)	27.62	29.55	39.34	46.13	8.36
Eicosadienoic acid (C20:2, n-6)	2.45	2.45	2.78	3.28	3.23
Heneicosylic acid (C21:0)	0.97	0.93	1.01	0.92	
Dihomo-γ-linoleic acid (C20:3, n-6)	0.28	0.28	0.57	0.78	
Arachidonic acid (C20:4, n-6)	0.49	0.67	0.90	0.82	0.83
Dihomo-γ-linolenic acid (C20:3, n-3)	0.16	0.44	0.69	0.85	
Behenic acid (C22:0)	0.30	0.38	0.39	0.46	0.79
Eicosapentaenoic acid (C20:5, n-3)	4.07	5.33	8.41	10.19	11.03
Erucic acid (C22:1, n-9)	0.77	0.84	0.94	1.34	
Tricosylic acid (C23:0)	0.27	0.31	0.36	0.44	
Adrenic acid (C22:4, n-6)	0.38	0.85	1.57	1.87	
Lignoceric acid (C24:0)	0.20	0.25	0.47	0.49	
Clupanodic acid (C22:5, n-3)	5.23	6.33	9.79	11.80	
Nervonic acid (C24:1, n-9)	0.55	0.59	0.73	0.80	
Docosahexaenoic acid (C22:6, n-3)	4.27	4.82	7.65	9.64	5.44
Total	3663.07	3799.54	4677.74	5086.11	1919.70
Saturated fatty acids	500.52	508.12	617.85	627.77	430.61
Monounsaturated fatty acids	1420.54	1520.29	1997.49	2299.54	263.47
Polyunsaturated fatty acids	1742.01	1771.13	2062.40	2158.80	1225.61
Polyunsaturated fatty acids (n-3)	255.08	273.39	361.45	414.21	306.53
Polyunsaturated fatty acids (n-6)	1486.93	1497.74	1700.95	1744.59	903.40
n-6/n-3	5.83	5.48	4.71	4.21	2.95

Values are expressed in mg/100 g. HCP: Hemp co-product; CLA: Conjugated linoleic acid; T: treatment; HCP: hemp co-product.

Table 4. Laying hen (Control, T3, T6, T9) performances (body weight, feed consumption, mortality, hen-day egg production, feed consumption and egg mass production).

	Control 0%	T_3 3%	T_6 6%	T_9 9%	SEM	*p*-value
Body weight 25th week (g)[a]	1839	1773	1800	1797	13.6	NS
Body weight 32nd week (g)[b]	1868	1802	1831	1840	15.6	NS
Feed consumption (g/hen/day)	122.3	118.7	118.3	120.7	0.73	NS
Mortality (%)	0	0	0	0	–	–
Hen-day egg production (%)	92.7[a]	89.1[b]	89.0[b]	94.6[a]	0.54	<0.001
Feed consumption (g/egg)	132.2	134.6	133.6	128.2	1.12	NS
Egg mass production (g/hen/day)	56.2[b]	53.7[c]	54.2[c]	59.0[a]	0.37	<0.001

Different superscript letters indicate significant different data ($p < 0.05$).
[a]24th week – 25th week: 2 weeks of adaptation.
[b]26th week – 32nd week: 7 weeks of experiment.
T:treatment; SEM: standard error of mean; NS: not significant.

Laying hens performances

As reported, the increasing inclusion of the hemp co-product in the diet of the laying hens did not result in changes in their final weight both following the adaptation period and at the end of the experimental trial. The same results were also observed for feed consumption (g/hen/day).

In parallel, the T_9 group (94.6%) reported a hen-day egg production comparable to that of the control

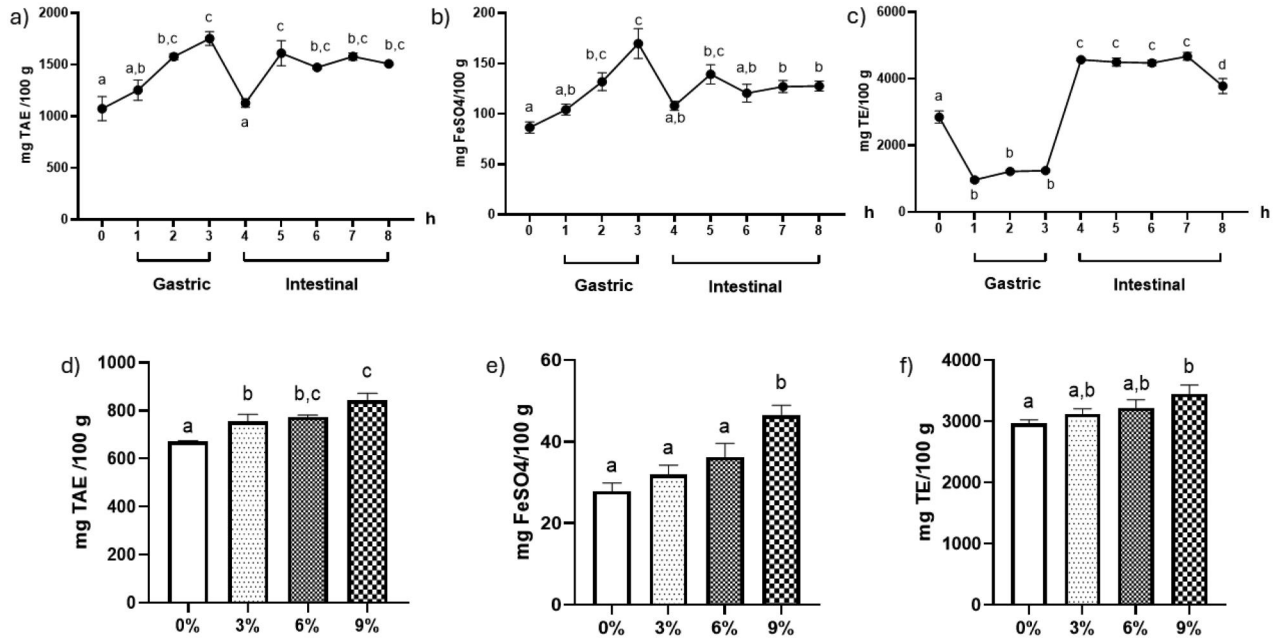

Figure 1. Total phenolic content and antioxidant activity (FRAP and ABTS) of hemp co-product and dietary treatments. TAE: Tannic acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicates. Different superscript letters indicate significant different data ($p < 0.05$). (a) Total phenolic content of hemp-co product monitored each hour; (b) FRAP assay of hemp-co product monitored each hour; (c) ABTS assay of hemp-co product monitored each hour, (d) Total phenolic content of dietary treatments; (e) FRAP assay of dietary treatments; (f) ABTS assay of dietary treatments; FRAP: ferric reducing antioxidant power; ABTS: 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

Table 5. Physical characteristic of eggs.

	Control 0%	T_3 3%	T_6 6%	T_9 9%	SEM	*p*-value
Egg surface (cm^2)	73.0^b	72.6^b	72.8^b	74.3^a	0.368	<0.001
Egg weight (g)	60.6^b	60.2^b	60.9^b	62.4^a	0.37	<0.001
Albumen weight (g)	40.6^b	40.2^b	40.6^b	42.2^a	0.17	<0.001
Yolk weight (g)	14.9	14.9	14.8	15.1	0.06	NS
Shell weight (g)	6.34	6.27	6.26	6.34	0.024	NS
Albumen percentage (%)	65.7^b	65.4^b	65.7^{ab}	66.3^a	0.10	0.015
Yolk percentage (%)	24.1	24.4	24.1	23.7	0.09	NS
Shell percentage (%)	10.3^a	10.2^a	10.2^a	10.0^b	0.03	0.006
Egg yolk/egg albumen (%)	36.8	37.4	36.8	35.9	0.20	NS
Shell thickness (μm)	375^a	370^{ab}	368^b	368^a	1.0	0.047
Shell deformity (mm)	0.29	0.29	0.28	0.29	0.0023	NS
Shell strength	4754	4769	4667	4744	32.2	NS
Shell strength (*N*)	48.5	48.6	47.6	48.4	0.33	NS
DSM yolk colour fan	4.48^d	5.14^c	5.72^b	6.35^a	0.045	<0.001
Redness (a*)^a	1.63^d	2.74^c	3.83^b	4.32^a	0.062	<0.001
Yellowness (b*)^b	36.9^d	42.5^c	46.7^b	48.7^a	0.27	<0.001

Different superscript letters indicate significant different data ($p < 0.05$).
[a]Redness (–100 green; 100 = red).
[b]Yellowness (–100 = blue; 100 = yellow).
T: treatment; SEM: standard error of mean; NS: not significant; DSM: Dutch State Mines.

(92.7%) and statistically superior ($p < 0.01$) to the T_3 (89.1%) and T_6 (89.0%), although feed consumption (g/egg) was similar between all dietary treatments. In addition, the 9% inclusion resulted in statistically ($p < 0.01$) higher egg mass production (59.0 g/hen/day) then 0%, 3% and 6% inclusion (56.2 g/hen/day, 53.7 g/hen/day and 54.2 g/hen/day, respectively).

Egg physical parameters

As shown in Table 5, the 9% inclusion of the hemp co-product showed a significantly ($p < 0.01$) greater egg surface (74.3 cm^2) and egg weight (62.4 g) than the control (73.0 cm^2; 60.6 g), 3% (72.6 cm^2; 60.2 g) and 6% inclusion (72.8 cm^2; 60.9 g), respectively.

Table 6. Fatty acid profile (mg/100 g) and cholesterol content (g/kg) of egg yolks.

	Control 0%	T_3 3%	T_6 6%	T_9 9%	SEM	*p*-value
Caproic acid (C6:0)	1.07^{c}	1.29^{b}	1.62^{a}	1.70^{a}	0.052	<0.001
Caprylic acid (C8:0)	2.48^{b}	2.62^{b}	2.95^{a}	2.70^{ab}	0.052	0.009
Capric acid (C10:0)	1.70^{b}	2.39^{a}	1.91^{b}	1.83^{b}	0.068	<0.001
Lauric acid (C12:0)	5.13^{b}	5.95^{a}	4.84^{c}	4.57^{d}	0.102	<0.001
Trydecylic acid (C13:0)	0.84^{d}	1.08^{c}	1.33^{b}	1.57^{a}	0.055	<0.001
Myristic acid (C14:0)	96.2^{a}	95.4^{a}	77.5^{b}	72.9^{b}	2.07	<0.001
Myristoleic acid (C14:1, n-5)	18.0^{a}	18.8^{a}	10.6^{b}	10.3^{b}	0.77	<0.001
Pentadecylic acid (C15:0)	18.5^{c}	20.9^{b}	26.5^{a}	25.1^{a}	0.63	<0.001
Cis-10–pentadecycli acid (C15:1, n-5)	12.3	13.4	12.7	13.2	0.23	NS
Palmitic acid (C16:0)	6311^{ab}	6379^{a}	6062^{b}	5511^{c}	74.8	<0.001
Palmitoleic acid (C16:1, n-7)	714^{a}	673^{b}	554^{c}	514^{d}	15.9	<0.001
Margaric acid (C17:0)	49.1^{b}	58.6^{a}	52.7^{b}	44.0^{c}	1.18	<0.001
Stearic acid (C18:0)	2233^{a}	2243^{a}	2262^{a}	1980^{b}	30.3	<0.001
Olenic acid (C18:1, n-9)	11453^{ab}	11988^{a}	11774^{a}	11036^{b}	118.0	0.018
Vaccenic acid (C18:1, n-7)	568^{a}	562^{a}	531^{b}	505^{b}	6.7	0.001
Linoleic acid (C18:2, n-6)	4078	4082	4374	4187	47.9	NS
Conjugated Linoleic Acid (CLA; 18:2 c9,t11)	5.02^{b}	5.86^{a}	6.29^{a}	5.04^{b}	0.122	<0.001
Conjugated Linoleic Acid (CLA; 18:2 t10,c12)	1.63^{c}	1.87^{b}	2.27^{a}	2.10^{a}	0.054	<0.001
γ-linoleic acid (C18:3, n-6)	32.0^{b}	30.6^{b}	41.9^{a}	41.6^{a}	1.07	<0.001
α-linolenic acid (C18:3, n-3)	319^{c}	315^{c}	367^{b}	418^{a}	8.4	<0.001
Arachidic acid (C20:0)	6.22^{b}	6.11^{bc}	8.26^{a}	5.60^{c}	0.204	<0.001
Gondoic acid (C 20:1, n-9)	73.1^{a}	65.8^{b}	72.2^{a}	63.5^{b}	1.06	<0.001
Eicosadienoic acid (C20:2, n-6)	44.2^{b}	50.8^{a}	50.0^{a}	42.7^{b}	0.86	<0.001
Heneicosylic acid (C21:0)	2.63^{c}	3.95^{b}	4.41^{a}	4.49^{a}	0.151	<0.001
Dihomo-γ-linoleic acid (C20:3, n-6)	25.8^{b}	27.1^{b}	33.4^{a}	33.1^{a}	0.70	<0.001
Arachidonic acid (C20:4, n-6)	505^{b}	559^{a}	581^{a}	515^{b}	8.5	0.001
Dihomo-γ-linolenic acid (C20:3, n-3)	4.97^{c}	5.72^{b}	5.63^{b}	7.26^{a}	0.168	<0.001
Juniperonic acid (C20:4, n-3)	4.51^{a}	3.85^{b}	3.62^{b}	4.18^{ab}	0.114	0.023
Behenic acid (C22:0)	3.86^{c}	4.40^{b}	5.04^{a}	4.64^{b}	0.095	<0.001
Eicosapentaenoic acid (C20:5, n-3)	5.05^{c}	6.43^{b}	7.08^{a}	6.09^{b}	0.153	<0.001
Erucic acid (C22:1, n-9)	3.31^{a}	3.56^{a}	3.74^{a}	2.39^{b}	0.128	<0.001
Docosadienoic acid (C22:2, n-6)	10.89^{a}	6.90^{d}	8.22^{b}	7.47^{c}	0.289	<0.001
Tricosylic acid (C23:0)	4.26^{b}	3.89^{b}	4.73^{a}	4.33^{ab}	0.089	0.005
Adrenic acid (C22:4, n-6)	8.1^{b}	11.6^{b}	33.6^{a}	33.0^{a}	2.25	<0.001
Lignoceric acid (C24:0)	4.03^{c}	5.51^{b}	6.16^{a}	5.65^{b}	0.157	<0.001
Clupanodic acid (C22:5, n-3)	51.1^{c}	54.4^{c}	89.6^{a}	74.1^{b}	2.93	<0.001
Nervonic acid (C24:1, n-9)	7.92^{a}	6.77^{b}	7.94^{a}	7.56^{a}	0.121	<0.001
Docosahexaenoic acid (C22:6, n-3)	335^{c}	459^{b}	542^{a}	521^{a}	15.7	<0.001
Total	27023^{ab}	27788^{a}	27636^{a}	25721^{b}	269.4	0.019
Saturated fatty acids	8742^{a}	8837^{a}	8524^{a}	7672^{b}	104.4	<0.001
Monounsaturated fatty acids	12849^{a}	13331^{a}	12966^{a}	12152^{b}	132.7	0.009
Polyunsaturated fatty acids	5431^{b}	5620^{b}	6145^{a}	5897^{ab}	76.6	0.002
Polyunsaturated fatty acids (n-3)	720^{c}	844^{b}	1015^{a}	1031^{a}	25.0	<0.001
Polyunsaturated fatty acids (n-6)	4705^{b}	4768^{b}	5122^{a}	4859^{ab}	57.4	0.044
n-6/n-3	6.54^{a}	5.65^{b}	5.05^{c}	4.72^{d}	0.127	<0.001
HH	2.61^{d}	2.70^{c}	2.89^{b}	3.00^{a}	0.029	<0.001
Atherogenic index	0.367^{a}	0.358^{b}	0.334^{c}	0.322^{d}	0.0034	<0.001
Thrombogenic index	0.790^{a}	0.753^{b}	0.696^{c}	0.653^{d}	0.0098	<0.001
Peroxidation index	38.2^{d}	41.6^{c}	47.2^{b}	48.2^{a}	0.76	<0.001
Cholesterol	11.4^{a}	10.7^{b}	10.4^{bc}	10.0^{c}	0.13	<0.001

Different superscript letters indicate significant different data ($p < 0.05$). CLA: Conjugated linoleic acid; HH: Hypocholesterolemic/hypercholesterolemic fatty acid ratio; T: treatment; SEM: standard error of mean; NS: not significant.

No differences were observed in the weight of each egg component, with the exception of albumen, with significantly ($p < 0.01$) greater values for T_9 (42.2 g) than control (40.6 g), T_3 (40.2 g) and T_6 (40.6 g). These values resulted in a significantly ($p < 0.05$) higher albumen percentage for T_9 (66.3%) than for control (65.7%) and T_3 (65.4%), but comparable to T_6 (65.7%).

Although no difference was observed for percentage of yolk, the percentage of control shell (10.3%) was similar to T_3 (10.2%) and T_6 (10.2%); all significantly ($p < 0.05$) superior to T_9 (10.0%). Despite this, the shell thickness, deformity and strength showed no appreciable difference between the groups.

Finally, as reported in Table 5, interesting results were obtained for egg yolks colour. More specifically, increasing levels of inclusion of hemp co-product resulted in significant ($p < 0.01$) rises between each group in the redness (1.63; 2.74; 3.83; 4.32) and yellowness (36.9; 42.5; 46.7; 48.7) of the yolks, for control, inclusion at 3%, 6% and 9%, respectively.

Nutritional and functional profile of egg yolks

The nutritional profile of egg yolks is shown in Table 6.

The 9% inclusion (7672 mg/100 g) of the hemp co-product resulted in a significant reduction ($p < 0.01$) in the SFAs content compared to the control (8742 mg/100 g), 3% (8837 mg/100 g) and 6% inclusion (8524 mg/100 g). The same trend ($p < 0.05$) was recorded for MUFAs (12849 mg/100 g; 13331 mg/100 g; 12966 mg/100 g; 12152 mg/100 g, for control, T_3, T_6 and T_9, respectively). Total PUFAs values were significantly ($p < 0.05$) higher for T_3 (6145 mg/100 g), compared to control (5431 mg/100 g) and T_6 (5620 mg/100 g), but highly comparable with T_9 (5897 mg/100 g). No difference was observed between control and inclusion at 3% and 9%. The same tendency was observed for n-6 PUFAs. In contrast, increasing inclusions of the hemp co-product increased the amount of n-3 PUFAs. More specifically, T_3 (844 mg/100 g) showed significantly ($p < 0.01$) higher values than the control (720 mg/100 g) and lower content than T_6 (1015 mg/100 g) and T_9 (1031 mg/100 g). No difference was recorded between T_6 and T_9. These results yielded an n-6/n-3 dependent on inclusion levels, with statistically significant differences ($p < 0.01$) between each group (6.54; 5.65; 5.05; 4.72, respectively for control, T_3, T_6 and T_9).

Table 7. Content of α-tocopherol, γ-tocopherol, retinol, β-carotene, lutein and zeaxanthin in egg yolks.

	Control 0%	T_3 3%	T_6 6%	T_9 9%	SEM	*p*-value
α-tocopherol	231	256	261	252	4.4	NS
γ-tocopherol	28.7^{c}	33.6^{b}	42.1^{a}	45.1^{a}	1.28	<0.001
Retinol	9.8	10.3	10.0	10.1	0.12	NS
Lutein	5.8^{d}	10.6^{c}	16.0^{b}	21.5^{a}	1.09	<0.001
Zeaxanthin	5.15	5.07	5.16	5.95	0.163	NS
β-carotene	0.0608^{a}	0.0605^{a}	0.0578^{ab}	0.0531^{b}	0.001	0.029

Values are expressed in mg/kg DM. Different superscript letters indicate significant different data ($p < 0.05$).
T: Treatment; SEM; standard error of mean; NS: not significant.

The same tendency was observed for the hypocholesterolemic/hypercholesterolemic fatty acid ratio, atherogenic, thrombogenic and peroxidability indices.

Finally, interesting results were obtained for cholesterol content. More specifically, the egg yolks of hens fed with 9% hemp co-product (10.0 g/kg) showed a similar level to the T_6 (10.4 g/kg) group, but significantly lower than T_3 (10.7 g/kg) and control (11.4 g/kg). At the same time, T_3 and T_6 were significantly higher than the control.

As reported in Table 7, although no difference was reported for α-tocopherol, the inclusion of the hemp co-product at 6% (42.1 mg/kg) and 9% (45.1 mg/kg) significantly increased ($p < 0.01$) the γ-tocopherol content compared to the control (28.7 mg/kg) and inclusion at 3% (33.6 mg/kg). Interesting results were reported for β-carotene and lutein values. In the first case, higher inclusions of hemp co-product corresponded to lower β-carotene values in egg yolks with higher values for the control (0.0608 mg/kg), although comparable to T_3 (0.0605 mg/kg), T_6 (0.0578 mg/kg), but significantly higher ($p < 0.05$) than T_9 (0.0531 mg/kg). In contrast, lutein content was dependent on hemp inclusion percentages, with significant ($p < 0.01$) differences between each group (5.8 mg/kg, 10.6 mg/kg, 16.0 mg/kg and 21.5 mg/kg, respectively for control, inclusion at 3%, 6% and 9%).

Following the green chemical extraction process, egg yolks from hens fed the hemp co-product had a significantly ($p < 0.05$) higher TPC (Figure 2a) than those receiving the basal diet (295.09 ± 22.18 mg TAE/100g; 458.25 ± 30.93 mg TAE/100g; 489.45 ± 38.10 mg TAE/100 g; 414.36 ± 24.53 mg TAE/100 g, respectively for control, T_3, T_6 and T_9).

This trend was also confirmed for the antioxidant activity measured by the ABTS method (Figure 2c). The FRAP assay (Figure 2b) showed that, although the control (38.65 ± 2.87 mg FeSO4/100 g) was comparable

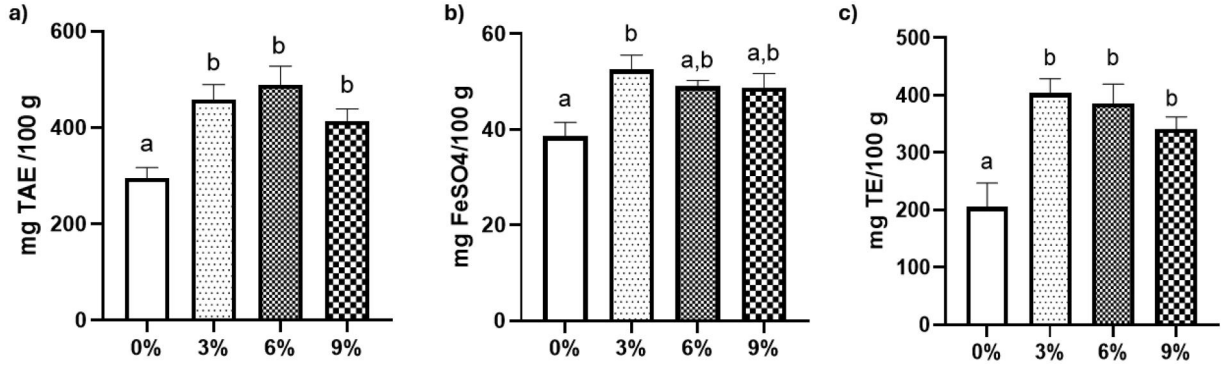

Figure 2. Total phenolic content and antioxidant activity (FRAP and ABTS) of egg yolks after green chemical extraction. TAE: Tannic acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicated. Different superscript letters indicate significant different data ($p < 0.05$). (a) Total phenolic content; (b) FRAP assay; (c) ABTS assay.

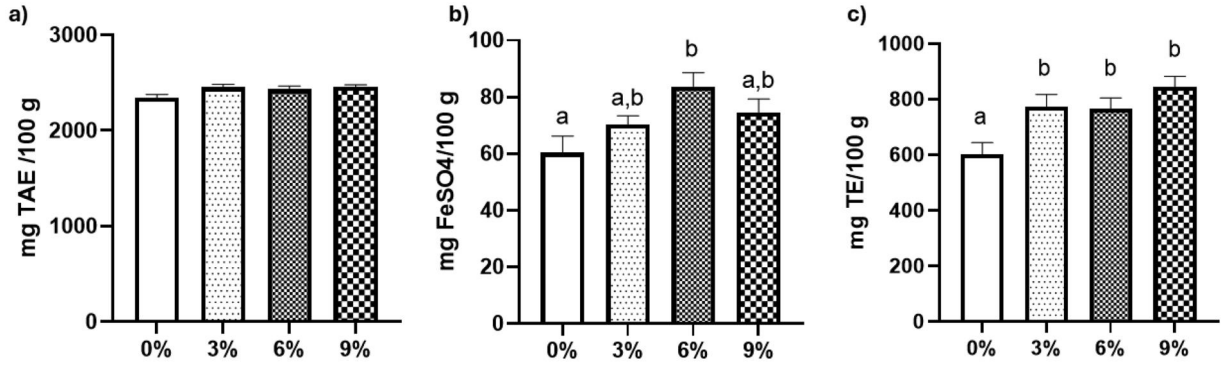

Figure 3. Total phenolic content and antioxidant activity (FRAP and ABTS) of egg yolks after *in vitro* digestion. TAE: Tannic acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicates. Different superscript letters indicate significant different data ($p < 0.05$). (a) Total phenolic content; (b) FRAP assay; (c) ABTS assay; FRAP: ferric reducing antioxidant power; ABTS: 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); SEM: standard error of mean.

to the inclusion at 6% (49.01 ± 1.26 mg FeSO4/100 g) and 9% (48.74 ± 2.97 mg FeSO4/100 g), it was significantly ($p < 0.05$) lower than 3% (52.53 ± 3.07 mg FeSO4/100 g), while no difference was noted between T_3, T_6 and T_9.

As shown in Figure 3, following the digestive process, although no difference was observed in the TPC (Figure 3a), the ABTS data confirmed what was previously reported (Figure 3c). More specifically, each inclusion of the hemp co-product recorded significantly ($p < 0.05$) higher values than the control (601.12 ± 42.40 mg TE/100 g; 773.17 ± 43.77 mg TE/100 g; 765.64 ± 38.71 mg TE/100 g; 843.48 ± 38.71 mg TE/100 g, for 0%, 3%, 6% and 9%, respectively). For FRAP (Figure 3b), higher results were obtained for T_6 (83.70 ± 4.92 mg FeSO4/100 g), comparable with T_3 (70.36 ± 3.06 mg FeSO4/100g) and T_9 (74.62 ± 4.65 mg FeSO4/100g), but significantly higher than the control (60.37 ± 5.85 mg FeSO4/100 g). No difference was observed between control, T_3 and T_9.

Discussion

Although it is difficult to compare the nutritional profile of the hemp co-product used in this trial with values found in the literature, mainly due to the harvesting method and processing, it is evident that it presents a high nutritional profile.

More precisely, it can be assumed that the interesting protein content derives from the contribution of HSs, hulls, stems and leaves, as reported in the literature (Kleinhenz et al. 2020; Vastolo et al. 2021; Ely and Fike 2022; Lanzoni et al. 2023b). At the same time, it is distinguished by a lipid profile typical of hemp-based matrices, characterised by a higher content of PUFAs, compared to MUFAs and SFAs (Callaway 2004; Vonapartis et al. 2015). More specifically, n-6 PUFAs present a higher percentage than n-3, resulting in an n-6/n-3 PUFAs (2.95) similar to that reported by Galasso et al. (2016) (2.8–4.5). However, as previously noted, the high ashes and especially the fibre content affected its digestibility. In spite of this, increasing inclusions of up to 9%, did not alter the digestibility of DM and protein in the diets, demonstrating its potential use.

At the same time, the hemp co-product showed an interesting functional profile. In particular, the behaviour of phenolic and antioxidant compounds was studied every hour to understand at what stage of digestion they are released and can benefit the animal health.

Precisely, as previously described, from the beginning of the oral phase, the co-product showed an increasing TPC reaching a peak at the end of the gastric phase (3 h).

This trend is confirmed in our previous study, in which hemp-based products (HSs, HSs protein, flowers) were digested with the same *in vitro* protocol (Lanzoni et al. 2023b). These results could be explained by the action of the acidic pH of the stomach, which causes the release of phenolic compounds as a result of the breaking of protein and polysaccharide bonds, particularly of fibre, the component with the highest phenol content (Goulas and Hadjisolomou 2019). Furthermore, as reported by Pineda-Vadillo et al. (2016), the stomach's pH is able to protect the structure of the phenols, enabling their exact quantification.

Later, following the addition of pancreatin (4 h), a steep decrease was recorded. As explained by Wojtunik-Kulesza et al. (2020), this is a consequence of the instability of phenols at alkaline pH, particularly during the pancreatic phase. More precisely, this condition causes a structural reorganisation of phenols compounds that modulates their function and bioaccessibility.

However, as previously described, the final TPC showed values comparable to those at the end of the gastric phase. As demonstrated by Tarko et al. (2013), only phenols that are released from the food matrix during the digestive process and reach the gut can be absorbed (Tarko et al. 2013). Although 10% of these compounds remain bound to the food matrix, approximately 48% are digested in the small and 42% in the large intestine (Tarko et al. 2013).

This is of paramount importance, as their absorption can exert multiple positive functions in the body, such as antioxidant, anti-inflammatory and improving intestinal health (Mahfuz et al. 2021).

As just reported, phenols are characterised by antioxidant activity. In support of this, the FRAP assay showed the same trend as the TPC.

However, the ABTS method reported a completely opposite course. As described by Zhou et al. (2016), such differences between the antioxidant assays can be observed as the sample contains a high antioxidant profile with different molecules with diverse mechanisms of action. As shown, higher values were obtained in the ABTS assay.

This is in line with the content of α,γ-tocopherol, lutein, β-carotene, and zeaxanthin observed in the hemp co-product. More precisely, as reported by Farinon et al. (2020), tocopherols are fat-soluble compounds with a high antioxidant capacity that can protect the lipid fraction from oxidation due to their high ability to scavenge free radicals, the mechanism of action of ABTS. Of these, as described by Porto et al. (2015), γ-tocopherol has been indicated as the most active antioxidant in lipids and consequently is the one that most contributes, together with other antioxidant compounds (e.g. phenols), to providing high oxidative stability in hemp-based products. At the same time, the hemp co-product showed good carotenoid values, with higher concentrations for lutein, β-carotene and zeaxanthin respectively, confirming the observations of Farinon et al. (2020). Again, these compounds are characterised by a greater free radical scavenging mode of action than metal chelation, the mechanism of action of FRAP assay (Sindhu et al. 2010).

As shown so far, the high functional properties of the hemp co-product have improved that of diets, especially T_9, demonstrating that the increased inclusion not only preserves digestibility parameters, but also increases the functional value.

These results can be correlated with the performance of the treated animals.

As previously described, the body weight of the treated animals showed no difference during both the adaptation period and the entire trial.

This finding confirms both the value of the diets digestibility and the possibility of including the co-product up to 9% without any negative aspects for the growth performances of the animals, despite higher values for ashes and fibre content than the control. This result is most probably related to the age of the treated animals (21 weeks). In this period, chickens are characterised by a developed gastrointestinal system. However, as reported by Vispute et al. (2019), in the early stages of the production cycle (0–3 weeks), it would be better to avoid the use of hemp-based matrices, as at this stage the chickens have a poorly formed mucosa, which is not able to produce enzymes to degrade cellulose. This was also observed by Gakhar et al. (2012) in laying hens.

A key parameter in the laying hen sector is certainly hen-day egg production (Lanzoni et al. 2023a). Regarding this, as previously reported, the 9% inclusion resulted in better results than T_3 and T_6, but similar to the control. For this reason, it can be deduced that the effect observed with the inclusion of the co-product at 3% and 6% is transitory and probably not specifically related to the dietary treatment. Indeed, it is important to emphasise that results regarding hen-day egg production are often discordant in the literature. Although, Skřivan et al. (2019) observed that the inclusion of 3% HSs in the diets of laying hens significantly improved the laying rate (93.6%), compared to the control (88.7%) and the inclusion of 6% (86.40%) and 9% (89.40%), Gakhar et al. (2012) observed no difference up to an inclusion of HSs of 20%. This was confirmed also by Neijat et al. (2016) with an HSs inclusion of 30%. This trend was also registered with the use of hemp co-products. In particular, the inclusion of 20% and 30% HSs in the diets of laying hens showed no change as demonstrated by Silversides and Lefrançois (2005) and Rajasekhar et al. (2021), respectively.

Finally, T_9 had a significantly higher egg mass production (g/hen/day) than the control. However, the latter was greater than T_3 and T_6. Again, it is possible that the effect observed for the inclusion of T_3 and T_6 is not related to dietary treatment. Indeed, as reported by Halle and Schöne (2013), the inclusion of 15% HSs cake in the diet of hens did not increase egg mass production. This was also confirmed by Gakhar et al. (2012) with up to 20% HSs.

For this reason, although better production performances was obtained in this study with the inclusion of 9% hemp co-product, further investigation is needed to understand the correlation between dietary

treatment, deposition hen/day egg production and egg mass production.

As previously reported, interesting results were also obtained for egg physical parameters and nutritional/functional profile of egg yolks.

As observed, the 9% inclusion of the hemp co-product resulted in significantly higher egg surface and weight than the other treatments, partially confirming what was described by Mierliță (2019). More specifically, the authors observed how the inclusion of 8% HSs in the diet of laying hens resulted in increased egg weight compared to the control (based on soybean meal). However, this trend was not reported by Konca et al. (2019) and Silversides and Lefrançois (2005) with an inclusion of 15% HSs and up to 20% HSs cake, respectively. Although, Johnston and Gous (2007) reported an increase in weight to be correlated with an increase in yolk weight, in this study the only increment was found to be egg albumen, confirming the findings of Halle and Schöne (2013).

Albeit shell thickness was negatively affected by the increase in hemp co-products inclusions, other parameters such as deformity, strength and weight were not altered. The latter plays a key role. This result is in agreement with the similar values of calcium and phosphorus in the different dietary treatments observed in Table 2. As reported by Keshavarz (1994), shell formation is influenced by the content of these two minerals in the diets, which are able to regulate the pH of the blood, consequently affecting its acid-base balance. More specifically, maintaining this equilibrium plays a key role in bicarbonate homeostasis and subsequent shell formation (Keshavarz 1994). However, as shown by Neijat et al. (2014), it is difficult to find a direct link between dietary treatment and the content of these minerals in the blood; in fact, as reported in the literature, the inclusion of hemp products in the diets of laying hens has yielded discordant results. While Mierlita et al. (2024) and Park et al. (2014) observed no difference, Skřivan et al. (2019) showed a negative correlation as HSs inclusion increased. Finally, another parameter investigated in the literature concerns the colour of the yolks, although it is difficult to identify it as a quality parameter, as it depends a lot on individual preferences (Lanzoni et al. 2023a). As reported in Table 5, increasing inclusions of hemp co-product resulted in increasing redness and yellowness values, confirming what was observed by Goldberg et al. (2012), Park et al. (2014), Skřivan et al. (2019) and Konca et al. (2019). As argued by Goldberg et al. (2012), colour intensity is influenced by the presence of carotenoids. More specifically, in this study, observing the values reported in Table 7, it is most likely, that the differences observed are due to the lutein content, rather than β-carotene and zeaxanthin, confirming what Kasula et al. (2021) reported.

From a nutritional point of view, it is interesting to observe how the inclusion of the hemp co-product modulated the lipid profile of egg yolks.

More specifically, although there would seem to be no correlation between different dietary treatments and total lipid content, the different fractions (SFAs, MUFAs, PUFAs) were highly modified. In particular, T_9 showed a significantly reduced SFAs content compared to the control, T_3 and T_6, mainly due to the reduction of myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0). This result is most likely also related to the digestive physiology of chickens. In fact, as reported by Ravindran et al. (2016), fatty acids released during digestion differ in their ability to form micelles and then be absorbed, with unsaturated ones characterised by a greater ease of formation than SFAs, especially long-chain ones. As argued by the authors, it is believed that the presence of even just one double bond is sufficient to increase the ease of micelle formation, due to a greater bending of the three-dimensional structure. In addition, unsaturated fatty acids are able to function as natural emulsifiers favouring this process. These would favour a higher absorption and consequently a higher presence in the egg yolks (Ravindran et al. 2016).

This trend, was also observed for the MUFAs content, confirming the values of Shahid et al. (2015), Konca et al. (2019), and Mierlita et al. (2024).

In general, and also in this study, these results are due to the fact that hemp-based products are characterised by higher values of PUFAs than SFAs and MUFAs (Shahid et al. 2015). In particular, for n-3 and n-6 PUFAs in egg yolks, better results were observed with the inclusion of 6% and 9% of the co-product, partly reflecting the dietary content.

Within the n-3 and n-6 PUFAs, it is important to assess the content of linoleic acid (C18:2, n-6) and α-linolenic acid (C18:3, n-3), the main fatty acids of the hemp co-product. More specifically, although no differences were shown for the content of linoleic acid, T_9 showed a significantly higher α-linolenic acid content than the control, T_3 and T_6. This result plays a key role as these two fatty acids are considered essential since they cannot be synthesised by the human metabolism but have to be introduced through the diet (Farinon et al. 2020).

In parallel, they are the precursors of biologically active n-6 and n-3 PUFAs in humans. These include

long-chain arachidonic acid (20:4, n-6) derived from the conversion of linoleic acid, docosahexaenoic acid (22:6-n3) and eicosapentaenoic acid (20:5, n-3) both obtained from the precursor α-linolenic acid (Lanzoni et al. 2023a). Indeed, in this study, arachidonic acid levels were significantly increased in eggs compared to dietary treatments, with better results for T_6. However, for eicosapentaenoic acid this trend was not observed. More specifically, although the inclusion of 6% allowed differences to be observed compared to the other dietary treatments, the values for T_6 and T_9 in eggs were lower than those in diets. This result may be explained because α-linolenic acid and linoleic acid compete for the same enzyme (Δ6 desaturase) that converts α-linolenic acid to eicosapentaenoic acid and linoleic acid to arachidonic acid, leading to a reduction in the synthesis of one of the two fatty acids (Shahid et al. 2015).

At the same time, T_6 and T_9 reported a significantly higher value for docosahexaenoic acid than the control and T_3, confirming what has been reported above. The increase of these biologically active forms in egg yolks is of great importance, as they play major roles in the maintenance of cell membrane structures, cardiovascular health, development of neurological functions, obesity and inflammatory processes (Farinon et al. 2020).

In addition to the evaluation of PUFAs, as reported by Simopoulos (2008), it is important to analyse the n-6/n-3 ratio, as this is a fundamental index involved in maintaining an optimal state of health, particularly in preventing the onset of chronic degenerative diseases. In this study, as noted earlier, the increased inclusion of the hemp co-product reduced the n-6/n-3 ratio, leading to a better result for T_9. Indeed, as pointed out by the European Food and Safety Authority (EFSA 2009), the ideal n-6/n-3 ratio should be approximately 3.5; a value typical of diets, including the Mediterranean diet, that are poorly associated with coronary heart disease (Callaway 2004).

In parallel, the evaluation of the ratio of hypocholestorolaemic/hypercholesterolaemic (h/H) fatty acids showed significantly better results with increasing inclusion of the hemp co-product. As reported by Chen and Liu (2020), for human health, higher values are considered more beneficial due to the effects of hypocholestorolaemic fatty acids (PUFAs and C18:1) on cholesterol metabolism.

This trend was also confirmed for the atherogenic and thrombogenic indices. They describe the relationship between pro-atherogenic (saturated) and anti-atherogenic (unsaturated) fatty acids and the tendency of fatty acids to form clots within blood vessels, respectively (Chen and Liu 2020). Although all groups reported values below the upper limits (0.5 and 1, respectively), increasing inclusions of the hemp co-product were found to be positively associated with improvements in these indices, confirming what was observed by Mierlita et al. (2024).

As expected, the peroxidation index of the eggs showed higher values as the co-product inclusion levels increased, confirming a greater risk of oxidation. This effect is mainly due to the high content of n-3 PUFAs, which are highly susceptible to oxidation, confirming the above. Despite this, although not investigated in this study, products obtained after treatment with hemp-based products showed a lower content of Malondialdehyde, a marker of lipid peroxidation, than the control (Mierlita et al. 2024; Mierliță 2019). As explained by the authors, this effect is due to the high levels of antioxidant molecules, in particular tocopherols, that are able to prevent lipid peroxidation, a result also obtained in this study, as later discussed.

Finally, increasing concentrations of hemp co-product reduced cholesterol levels respectively. Although Mierliță (2019) reported no difference in cholesterol content in the egg yolks of hens fed HSs (80 g/kg and 200 g/kg), Shahid et al. (2015) observed a different trend. Specifically, the authors showed how increasing inclusions of HSs (150, 200 and 250 g/kg) significantly reduced the cholesterol content (16.91 ± 0.01 mg/g; 14.29 ± 0.01 mg/g; 11.65 ± 0.01 mg/g) compared to the control (19.27 ± 0.01 mg/g), respectively. Identical trend observed by Mahmoudi et al. (2015), Skřivan et al. (2019) and Vispute et al. (2019). This positive result can be related both to the presence of phytosterols in hemp-based products and to the peculiarities of the chickens' digestive process. In the first case, as argued by Shahid et al. (2015), the presence of phytosterols in HSs, in particular β-sitosterol, is able to reduce hypercholesterolaemia by absorbing cholesterol through processes such as co-precipitation and crystallisation. At the same time, as phytosterols are less water-soluble than cholesterol, they are able to replace cholesterol from intestinal micelles. In the second case, as reported by Ravindran et al. (2016), within chicken enterocytes, fatty acids are re-esterified and combined with multiple molecules (lipoproteins and phospholipids) including cholesterol to form chylomicrons. From here, the chylomicrons are secreted directly into the portal circulation and transported to various tissues, in particular to the liver, where the lipids are used for the synthesis of various compounds needed by the organism, such as lipoproteins and phospholipids,

metabolised as an energy source or stored in the tissues as fat stores. This phenomenon suggests that a higher concentration of fatty acids (characteristic of diets with increasing levels of hemp co-product) allows for a greater interaction with cholesterol leading to its physiological reduction both in the body and then in the yolk of the eggs produced.

Reducing cholesterol content is an important factor, as it is responsible for chronic or degenerative disorders including cardiovascular disease, the leading cause of mortality and morbidity globally (Carrillo et al. 2012; Miranda et al. 2015).

In general, from a functional point of view, egg yolks are an important source of α-tocopherol (Skřivan et al. 2019). Although, as reported by Hansen et al. (2015), to increase α-tocopherol levels in egg yolks, it is necessary to act only on the dietary level, such an effect was not observed in this study.

However, γ-tocopherol values were significantly higher for T_3, T_6 and T_9, confirming the values reported by Skřivan et al. (2019) and Mierlita et al. (2024). This result plays a key role, since as shown by Guinaz et al. (2009), egg yolks are generally characterised by a reduced γ-tocopherol value, below detection limits. Furthermore, as reported by Wagner et al. (2004), although all isoforms of tocopherol are absorbed in the intestinal mucosa, excessive enrichment of α-tocopherol in the human diet results in a lower absorption rate. This effect, however, is not reported for the γ-isoform, making it available for important functional activities in human health (Wagner et al. 2004). Among these, in addition to its antioxidant properties, γ-tocopherol is characterised by anti-inflammatory properties (reduction of prostaglandin synthesis), anti-diabetic properties (supports the viability of β-pancreatic cells and insulin production during inflammation stages), prevents the onset of diseases such as atherosclerosis and hypertension, reduces the occurrence of tumours and finally, is positively involved in the prevention of Alzheimer's disease by protecting the brain against reactive nitrogen species (Saldeen and Saldeen 2005).

In parallel, carotenoid values showed discordant results.

More specifically, β-carotene was found to be inversely proportional to co-product inclusion levels. Although Skřivan et al. (2019) and Mierlita et al. (2024) observed different trends, this finding was confirmed by Taaifi et al. (2023). More specifically, the authors demonstrated how increasing inclusions of HSs (10%, 20% and 30%) reduced the β-carotene content, respectively. At the same time, lutein increased significantly with each inclusion of the hemp co-product, with an increment of 182.8%, 275.9% and 370.7% for T_3, T_6 and T_9 respectively, compared to the control. This effect, as reported by Gao et al. (2020) is most likely due to the high deposition efficiency of lutein in yolk compared to β-carotene. Furthermore, as described by Donsou et al. (2023), carotenoids such as β-carotene are precursors of vitamin A. After absorption, β,β-carotene 15,15′-dioxygenase oxidatively cleaves this carotenoid at the central 15,15′ double bond to produce one or two molecules of retinal (vitamin A aldehyde). Although this process increases the concentration of vitamin A in eggs, it decreases the β-carotene content, explaining the results obtained.

To study the availability of functional compounds, as previously reported, egg yolks were chemically extracted and digested using an *in vitro* protocol.

In the first case, the binary extraction (H_2O:EtOH; 50:50) observed a significant increase in TPC in all dietary treatments that received the inclusion of the hemp co-product. This trend, although not statistically significant, was also observed following the digestion process. To our knowledge, there are no studies in the literature quantifying TPC in eggs following dietary treatment with hemp-based matrices. Despite this, Benakmoum et al. (2013) and Untea et al. (2020) confirmed these findings, observing an increase in TPC following inclusion of dried tomato peel and bilberry and walnut leaves respectively. As reported by the authors, the increase in TPC is caused by the deposition in the yolk of those compounds characterised more by a lipophilic nature; compounds highly present in hemp-based products (Farinon et al. 2020; Untea et al. 2020). This effect is of paramount importance because. As previously described, phenols, in addition to having important antioxidant, anti-inflammatory and gut-health enhancing activity (Mahfuz et al. 2021), are characterised by atheroprotective activity, affecting cholesterol metabolism positively (Zanotti et al. 2015). The above, coupled with the reduced cholesterol levels shown previously, demonstrate the potential of this co-product to improve the healthiness of eggs for human consumption.

This trend was also recorded for antioxidant activity measured with the ABTS assay following both the chemical extraction and *in vitro* digestion. This result, in addition to proving the involvement of phenolic compounds in the modulation of antioxidant activity, may support previous evidence which reported that molecules such as α, γ-tocopherol, isoforms of vitamin E, are the main antioxidant compounds capable of

providing primary protection against lipid peroxidation caused by free radicals (Traber and Atkinson 2007).

In parallel, Kim et al. (2011) attributed to molecules such as zeaxanthin, but especially lutein (significantly higher in the yolks of eggs from hens that received the hemp co-product), the ability to scavenge hydroxyl and superoxide radicals by means of a bond that results in their stabilisation. These molecules, as demonstrated by Panasenko et al. (2000), are also capable of eliminating peroxynitrite, a molecule characterised by a high pro-oxidant activity against nucleic acids.

An increase in antioxidant activity was also recorded with the FRAP assay, although it is complicated to find a close correlation with the dietary treatment administered.

Conclusion

In conclusion, the inclusion of the hemp co-product did not alter the dry matter and protein digestibility of the diet, compared to the control, while improving its functional profile, with better results obtained for 9%. These results were confirmed for production performance. Although no differences were observed in the body weight of the treated animals at the end of the trial compared to the control group for each % inclusion, egg mass production (g/hen day) showed better trends with 9% of hemp co-product inclusion. In parallel, for the nutritional profile of the egg yolks, although T_3 and T_6 showed no differences compared to the control for the content of saturated and monounsaturated fatty acids, T_9 showed significant differences, also increasing the content of polyunsaturated fatty acids with a better n-6/n-3 ratio. This trend was also confirmed for atherogenic, thrombogenic and peroxidation indices, as well as for cholesterol; factors negatively involved in the genesis of cardiovascular pathologies. Finally, following *in vitro* digestion, all inclusion levels of the co-product increased the antioxidant activity (ABTS) of the yolks of eggs capable of providing a healthier product for human consumption. Although these results support the valorisation of hemp co-products in the diet of laying hens, with better results with 9% inclusion, it is crucial to emphasise that the high variability of the co-products due to the genotype considered, environmental factors, agro-ecological conditions, harvesting techniques, but above all transformation processes represent a critical point in their utilisation, which may affect the repeatability of the results.

Acknowledgements

The authors would like to thank the Ministry of Agriculture of the Czech Republic (institutional support MZE-RO0723).

Ethical approval

The procedures performed with animals were in accordance with the Ethics Committee of the Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic (Prague, Czech Republic) and carried out in accordance with Directive 2010/63/EU for animal experiments. The protocol of this experiment was approved by the Ethics Committee of the Institute of Animal Science (Prague-Uhříněves, Czech Republic), file number 01/2023.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was funded by University of Milan "My first SEED Grant 2023", project n.23-UNIMI-MFSG-066 HappyHens.

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Data availability statement

The dataset generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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