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Evaluation of hemp cake (*Cannabis sativa*) and other hemp by-products of Greek origin and efficacy in dairy cow nutrition

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ABSTRACT

Initially, the nutritional value of the three main by-products of *Cannabis sativa* (hempseed cake, second-rate quality hemp seed and hemp hay), cultivated under Greek conditions and collected from four plantations in the Macedonian region was estimated. A second part of the study was the investigation of the effects of diet inclusion with hempseed cake (HSC) on the performance of Holstein dairy cows. The HSC used for this experiment was the one analyzed in the first part of the study. A total number of 20 lactating cows were allocated into two equal groups in a randomized block design. Cows of both groups were offered a total mixed ration (TMR) on the same feed allowance. The diet of the experimental group was formulated with 3.5 % hemp cake, at a quantity of 1kg of hemp cake per cow per day. Milk yield was recorded individually, and feed refusals were recorded on a pen basis daily, during the first 40 days of lactation. Individual milk samples were analyzed for their chemical composition, lipid oxidative stability and fatty acid composition and tetrahydrocannabinol (THC) and cannabidiol (CBD) content, as well as feed samples of each group. Individual blood samples were received for biochemical indices assessment. All data were subjected ANOVA statistical analysis. The results of hemp by-products chemical profile showed similar nutritional values among the different cultivation regions. The experimental results indicated that the inclusion of HSC did not affect milk production and composition ($P > 0.05$). However, diet inclusion with HSC favorably influenced milk fatty acid profile. Finally, serum NEFA concentration was lower for the HSC group while serum urea levels were higher ($P < 0.05$).

Keywords: Cannabis Sativa; Hempseed cake; Hemp by-products; dairy milk yield; fatty acid profile

INTRODUCTION

Industrial hemp *(Cannabis sativa L.),* commonly described as "multifactorial plant", is currently cultivated to offer high quality raw materials; serving fiber, food, oil, medicine and even biomaterials production (Pavlovic et al., 2019). Worldwide hemp production is increasing considerably the last decade, while in the European continent arable land available for this purpose has expanded by 70%, in a short period of five years (Bailoni et al., 2021). This increase in hemp production led to the inevitable increased availability of hemp by-products, thus creating the need for effective channelling (Semwogerere et al., 2020). Concurrently, the livestock sector is under continuous search for alternative feed resources that can offer financial and environmental stability, due to the intense fluctuation of conventional feedstuff prices and the increasing concerns regarding their ecological sustainability. The incorporation of hemp by-products in dairy cows' diets could alleviate both ongoing risks.

Hemp cultivation has many advantages among other widely grown crops, such as cotton and corn. In particular, hemp is an annual plant with significant fast growth potential that can operate efficiently as a CO_2 biomass converter (Rehman et al., 2021). Even more, its cultivation can take place in pesticide free conditions (Rehman et al., 2021). Regarding its ambient temperature requirements, it is considered as a thermophilic plant ideal for moderate climate zones, similar to the Mediterranean basin (Visković et al., 2023). Historically, in Greece, industrial hemp was thriving in the agricultural sector during the 20th century, before its legal prohibition. From 2016 and up today, in Greece, hemp cultivation has rejuvenated and various cultivars are growing, with Futura 75 being the dominant one during the years 2019 and 2020, mainly for the production of flour and seeds (Aboagye et al., 2018). As animal feed, industrial hemp can be used in the following 3 forms of by-products; second-rate quality seed, cake; and as hay either from the whole plant or from its upper part.

The European Food Safety Authority provides limits regarding the addition of industrial hemp by-products in animal diets. Specifically, in ruminant diets hemp use is considered safe up to 5 % as hemp seeds or hempseed cake, while hemp hay incorporation in the diet is suggested to range between 0.5 to 1.5 kg of plant dry matter (DM) (EFSA, 2011). The use of industrial hemp in animal nutrition is not widely investigated, yet. Despite the literature reports on the existence of antiquality agents such as phytic acid, tannins, and trypsin inhibitors (Russo, 2013), all three forms had high nutritive value and also secondary metabolites that may positively impact animal and human health. More specifically, hemp seeds contain 25 – 33 % crude protein (CP) and 26 – 38 % fat, rich in polysaturated fatty acids (PUFAs). Supplementary, hempseed cake is rich in rumen undegradable protein with satisfactory amino acid profile, while the total fat content ranges between 5 - 12 %, depending on the method of oil extraction (Mustafa et al., 1999).

In the available literature the effects of hempseed cake on dairy ruminants are not coherent, yet beneficial results on milk yield and composition have been reported. In a study with dairy cows fed with corn silage (494 g/ kg DM) and concentrates (506 g/kg DM), the addition of 143 g/kg DM hempseed cake led to increased milk yield while the addition of 233 and 318 g/kg DM had no significant effect in milk production (Karlsson et al., 2010). Milk yield and protein synthesis were improved in dairy ewes offered hempseed cake at 0.48 g daily, this effect was attribute to hempseed cake amino acid profile; balanced for milk production. In the same study ewes had significantly increased milk fat yield (61.13 vs 54.02 g per day) and higher polyunsaturated fatty acid content in milk (Mierliță et al., 2018).

The current literature emphasizes mostly on the effect of hemp by-products on milk yield. Also, important differences regarding their nutritional value are detected. Therefore, the first aim of the current study was to evaluate the nutritional components of the Futura 75 cultivar hemp by-products. The next aim was to investigate the effects of the inclusion of hempseed cake in dairy cows' diet on performance parameters, health status and milk organoleptic characteristics. The hypothesis of this experiment was that the addition of hempseed cake to the cows' diet will lead to improved milk production along with higher antioxidant activity and higher PUFAs content in the milk, as well as health status benefits.

MATERIALS AND METHODS

Ethic Guidelines of the Animal Research

This trial was carried out according to the regulations of the Greek Public Veterinary Service and approved by the Research Committee of Aristotle

University of Thessaloniki, under the project numbered 95918 with the name Green Feed. The health of the animals was monitored by a veterinary surgeon; animals were routinely vaccinated against Infectious Bovine Rhinotracheitis, *Escherichia Coli* K99, Rotavirus and Coronavirus before calving. All cows were free of *Brucella melitensis*. All procedures were performed by the same team members on all animals throughout the project.

Greek hemp samples collection

Futura 75, a French origin cultivar, is identified as the most productive and easiest cultivated in Greece resulting in higher preference by farmers, thus it was selected for further assessment in this study (Tsaliki et al., 2021). The seeds were imported in Greece from each farmer, according to European Union (EU) legislation, under strict control and monitoring by the Greek Ministry of Rural Development and Food. The cultivations were established in April and the harvesting dates were during September of 2020. The main available by-products from cultivated fields were hemp hay, and from hempseed oil production the hempseed cake as well as the second-rate quality seeds. Samples were collected from 4 regions of Macedonia; Western (Kozani), Central (Kalochori and Thermi) and East (Serres) after plant harvesting. From each region 10 subsamples of hemp hay, forming a final sample of 1 kg were collected. Similarly, 1 kg of hempseed cake and 1 kg of second-rate quality hemp seeds were collected from local industries of the above-mentioned areas.

Evaluation of hemp by-products chemical composition

Dry matter content was determined after drying 5 g of each sample at 100 °C for 5 hours. Organic matter was determined by drying 2 g of each sample, weighted in porcelain crucibles and placed in a furnace preheated to 600 °C for 12 h. AOAC method was applied to determine nitrogen (N) content of the samples. CP was then calculated by multiplying the N content by 6.25 (Horwitz et al., 2002). For the determination of NDF, ADF, ADL 100 g of each sample were dried for 48 h at 60 °C. After that, the samples were grinded to pass through a 1mm screen and 0.5 g of each sample was transferred into heat sealed filter bags of 25 microns. For neutral detergent fiber (NDF) and acid detergent fiber (ADF) determination, ANKOM 220 fiber analyzer (Ankom Technology, NY, USA) was used with amylase, and sulphite addition (Van Soest, Robertson, and Lewis 1991). ADF analysis was sequential to NDF analysis. ADF samples were incubated with 70% sulphuric acid. Acid detergent lignin (ADL) was estimated by the method of Goering and Van Soest (Goering and Van Soest, 1970).

Experimental Design and Feeding

The experiment was conducted, from January to March 2021, in a commercial dairy farm located in Kosmion, Komotini, Northern Greece. For this study, 20 purebred multiparous Holstein dairy cows were randomly allocated in two groups, balanced for body condition score, age and milk production, control and hemp treatment, for a period of 40 days, following 15 days of adaption period. All the animals were 21 days post-partum at the beginning of the experiment. The experimental diets were a conventional diet (CON) and a diet consisting furtherly of 1kg hempseed cake (HSC), in substitution of whole cottonseed and soybean meal (Table 1).

Table 1. Ration ingredients (kg/cow/day) and chemical composition of conventional and hempseed cake (HSC) diets (g/kg DM basis)

Ingredient	CON	HSC
Corn silage	33.00	33.00
Luzerne hay	3.60	3.60
Corn grain	6.50	6.50
Soybean meal 47%	4.00	3.70
Whole Cottonseed	1.25	0.55
Bypass fat	0.29	0.29
Sugar beet pulp	0.80	0.80
Molasses	0.80	0.80
Water	8.90	8.90
Limestone	0.50	0.50
Sodium bicarbonate	0.17	0.17
Vitamin and mineral premix[1]	0.30	0.30
Hemp Cake 24%	0.00	1.00
Dry Matter per cow (kg)	25.90	26.01
Chemical composition		
Crude Protein g/kg DM	140.66	138.76
Ether extract g/kg DM	47.40	46.59
Crude fiber g/kg DM	145.08	148.32
PDI g/kg DM	84.42	83.48
PDIA g/kg DM	39.60	38.54
NDF g/kg DM	285.23	293.12
ADF g/kg DM	153.75	155.45
ADL g/kg DM	26.19	29.23
Starch g/kg DM	169.97	169.84
Sugars g/kg DM	64.00	65.00
UFL/kg DM	0.98	0.98

[1] Vitamin and mineral mix contained (per kg DM of concentrate): 9000 IU of vitamin A; 85 mg of vitamin E; 3000 IU of vitamin D3; 1.5 mg/kg biotin; 6 mg/kg niacin; 45 mg/kg choline; 0.2 mg Co; 3 mg I; 100 mg/kg Fe; 50 mg Mn; 0,45 mg Se; 150 mg Zn; 6 g of

NaCl; 4 g of sulphur; 10 g of magnesium oxide; 15 g of monocalcium phosphate and 21 g of limestone.

The two rations were isoenergetic and isonitrogenous and were offered twice daily in the form of total mixed ration, while water was available *ad libitum*. Animals were group-fed following a constant feed allowance. Milking was carried twice daily, at 06:00 and 17:00 and individual milk yields were recorded at every milking. Individual milk samples were analyzed for total fat, total protein, lactose, total solids, solid-not-fat (SNF) by means of near-infrared spectroscopy using a MilkoScan 4000 (FOSS Electric, Integrated Milk TestingTM, Hillerød, Denmark) and somatic cell counts (SCC) were determined using a Fossomatic 5000 Basic (FOSS Electric, Denmark). An aliquot of each milk sample was kept at the refrigerator for further analysis.

Determination of the Fatty Acid Composition in Milk and Cannabis by-products

For the determination of fatty acid methyl esters in both milk and hemp by-products the frozen individual milk samples and hemp samples were prepared according to the method proposed by O'Fallon et al. (O'Fallon et al., 2007). Fatty acids were quantified by peak area measurement and the results were expressed as percentage (%) of the total peak areas for all quantified acids.

Determination of milk oxidative stability

Lipid oxidation was determined in individual milk samples collected the final day of the trial. Samples were preserved at cooling conditions and analyzed at days 1, 3 and 5 after their collection, following the modified spectrophotometric method (Ahn et al. 1992; 1998). According to the method, 500 μL of each sample was homogenized in 1.5 mL of distilled water with Ultra-Turrax T25 (Janke & Kunkel, IKA Labortechnik) for 15 sec. Then 1 mL aliquot was vortex mixed in the presence of 10 mL of butylated hydroxyanisol (7.2%) and 5 mL of thiobarbituric-trichloroacetic acid solution (20 mM TBA in 15% trichloroacetic acid). Afterwards, the samples were maintained in boiling water for 15 mins. Then, the cooled samples were centrifuged at 1000 × g for 15 min and the absorbance of each supernatant was measured at 532 nm with a spectrophotometer (UV 1700 PharmaSpec, Shimadzu, Japan). The concentration of malondialdehyde (MDA) in the samples was compared to a standard calibration curve and results were expressed in μmol MDA/L serum (ng/ml milk).

Determination of Blood Serum Parameters

Blood samples were obtained from each cow individually by jugular venipuncture into 10mL vacuum tubes without anticoagulant with a needle, on the first and last day of the experiment and analyzed individually. Samples were centrifuged (1600 × g for 15 min in 4 °C) and the collected serum was preserved at -20 °C until analysis. The following biochemical parameters were evaluated in the serum samples: total proteins, albumins, glucose, blood urea nitrogen (BUN), creatine phosphokinase (CPK) and gamma-glutamyl transpeptidase (γ-Gt) and they were assayed using an automated analyzer (TARGA CLIN/CHEM Analyzer, BT 1500 Biotechnica instruments Roma, Italy). Serum non-esterified fatty acids (NEFA) concentrations were measured using a commercial kit (NEFA FS, DiaSys Diagnostics Systems GmbH, Holzheim Germany), while β-Hydroxybutyrate acid (BHBA) was also measured by a commercial kit.

Determination of diet and milk THC-CBD-Terpenes content

The THC levels in milk and forage materials were determined by gas chromatography, a method which is described in commission implementing regulation (EU) No 809/2014, article 32, paragraph 6 of EU regulation No 1307/2013 (Pertwee 2006). According to this method the samples, except milk, were dried and grinded until the moisture reaches 8 - 13% (in our experiment we used a Vacuo at 70 °C for 48 h) and 1 mm diameter.

Statistical Analysis

Data were analyzed by ANOVA in the general linear model of the SPSS 25.00 statistical package (SPSS Inc., Chicago, IL). Individual cows were considered the statistical unit for the analysis. Data on total milk yield, milk characteristics and serum biochemical parameters were analyzed by means of one-way ANOVA. The homogeneity of the variances was tested using the Levene test. Significant differences were declared at a probability level of $P < 0.05$ between the experimental treatments, when a significant effect of treatment was detected by means of the ANOVA.

RESULTS

Hemp products chemical analysis.

The chemical composition of hemp by-products is detailed presented in Table 2. Hempseed cake showed similar nutritional values among the four different areas of collection. Crude protein mean value was 21.5 % and the highest CP was observed at Kalochori_Fut.25 samples while the lowest at Thermi_Fut25. The mean crude fat (CF) concentration was 8.27 % and Serres_Fut.25 showed the highest concentration of CF while Kalochori_Fut.25

the lowest. Mean crude fiber was 33.62 % and Thermi_Fut25 37.2 % had the highest values, while Kalochori_Fut. 25 had the lowest. NDF and ADF mean values were 48.2 % and 35.02 % accordingly (highest: Kalochori_Fut.25 49.4 % NDF and Kozani_Fut25 37.2 % ADF). Hemp hay highest CP and CF were observed at Serres_Fut.25 and Kalochori_Fut.25 showed the lowest. Finally, second-rate quality seeds CP and CF was higher at Thermi_Fut25 samples and lower at Kozani_Fut25.

Table 2. Evaluation of hemp products chemical composition on % DM basis

Hempseed cake	**Kozani_Fut. 25**	**Serres_Fut. 25**	**Kalochori_Fut. 25**	**Thermi_Fut. 25**	**Mean**
Dry Matter	90.8	93	91.3	92.2	91.82
Crude Protein	21.8	21.5	22.4	20.3	21.50
Crude Fat	8.2	8.6	7.8	8.5	8.27
Crude Fiber	33.2	35.2	28.9	37.2	33.62
NDF	48.3	47.3	49.4	47.8	48.20
ADF	37.2	34.2	33.6	35.1	35.02
ADL	11.6	11.8	12.5	11.4	11.82
Ash	5.3	5.95	5.5	5.8	5.63
Hemp hay					
Dry Matter	90.5	91.1	89.8	90.2	90.40
Crude Protein	17.1	19.8	16.6	17.8	17.82
Crude Fat	6.7	8.4	6.6	7.6	7.32
Crude Fiber	15.7	18.5	13.9	16.7	16.20
NDF	59.2	64.1	63.9	53.1	60.07
ADF	47.3	48.2	51.3	47.8	48.65
ADL	1.2	1.1	1.3	1.5	1.27
Ash	13.5	16.9	13.7	15.1	14.80
Hemp sq seed					
Dry matter	90.8	91.71	91.7	92.1	91.57
Crude Protein	16.4	17.64	16.9	18.1	17.26
Crude Fat	15.1	16.8	16.2	17.3	16.35
Crude Fiber	34.3	31.2	33.4	28.7	31.90
NDF	60	57.6	55.8	58.8	58.05
ADF	45	40.7	41.8	39.8	41.82
ADL	18.5	15.4	16.7	17.2	16.95
Ash	8.7	9.74	9.3	9.8	9.38

Milk yield and composition.

During the experimental period none of the animals showed any symptoms of disease. Milk yield followed the typical lactation curve in both groups and the incorporation of hempseed cake in HCS group did not affect milk yield ($P > 0.05$) (Table 3).

Table 3. Effect of hemp cake supplementation on milk yield

Milk yield (L/d)	Treatment			
	CON	**HSC**	**SEM[1]**	***P-value***
Day 1	34.86	34.75	0.810	0.948
Day 20	36.91	38.92	0.986	0.321
Day 40	34.55	35.77	0.880	0.503

[1]SEM: Standard Error of Mean.

Milk fat, protein, lactose, and total solids did not differ among the groups CON and HSC (P > 0.05) (Table 4), indicating that the inclusion of hempseed cake did not affect these parameters. However, HSC group had lower somatic cell counts present in milk samples compared to CON group (P = 0.005).

Table 4. Effect of hemp cake supplementation on milk composition

Milk composition	Treatment			
	CON	**HSC**	**SEM[1]**	***P-value***
Fat, %	3.43	3.73	0.132	0.279
Protein, %	3.33	3.39	0.062	0.679
Lactose, %	4.92	4.79	0.058	0.278
TS[2], %	10.38	12.22	0.661	0.301
SCC[3], $\times 10^3$/mL	134.20[a]	117.10[b]	14.557	0.005

[1]SEM: Standard Error of Mean. [2]TS: Total Solids. [3]SCC: Somatic Cell Counts. [ab]Different letters denote significant (P ≤ 0.05) differences between the dietary treatments.

Milk fatty acid composition and oxidation status

The HSC group (CON) had decreased pentadecanoic and palmitic fatty acids in contrast to CON (P < 0.05) (Table 5).

Table 5. Effect of hemp cake supplementation on milk fatty acid profile

	Treatment			
Fatty acids (%)	**CON**	**HSC**	**SEM[1]**	***P-value***
C6:0 (Caproic)	1.922	2.198	0.116	0.251
C8:0 (Caprylic)	1.845	1.858	0.075	0.933
C10:0 (Capric)	5.118	5.923	0.314	0.216
C11:0 (Undecanoic)	0.075	0.094	0.023	0.710
C12:0 (Lauric)	6.012	5.223	0.537	0.489
C14:0 (Myristic)	15.786	15.795	0.652	0.990
C14:1 (Myristoleic)	1.925	1.848	0.047	0.450
C15:0 (Pentadecanoic)	1.445[a]	0.934[b]	0.127	0.036
C15:1 (cis-10-Pentadecenoic)	0.143	0.136	0.026	0.898
C16:0 (Palmitic)	39.977[a]	36.630[b]	0.811	0.031
C16:1 (Palmitoleic)	2.030	2.086	0.116	0.822
C17:0 (Heptadecanoic)	0.415	0.301	0.036	0.115

[1]SEM: Standard Error of Mean. [ab]Different letters denote significant ($P \leq 0.05$) differences between the dietary treatments. [x,y] Values in the same row with different superscripts tend to differ ($0.05 < P \leq 0.10$).

Table 5. Continued

	Treatment			
Fatty acids (%)	**CON**	**HSC**	**SEM[1]**	***P-value***
C18:0 (Stearic)	7.446	7.255	0.244	0.716
C18:1n9t (Elaidic)	0.096	0.222	0.087	0.499
C18:1n9c (Oleic)	13.391[y]	15.893[x]	0.707	0.074
C18:2n6c (Linolelaidic)	1.960	2.394	0.286	0.475
C18:3n3 (α-Linolenic)	0.191	0.254	0.022	0.168
C20:1n9c (cis-11-Eicosenoic)	0.158	0.196	0.017	0.291
C20:4n6 (Arachidonic)	0.034[b]	0.193[a]	0.034	0.002

[1]SEM: Standard Error of Mean. [ab]Different letters denote significant ($P \leq 0.05$) differences between the dietary treatments. [x,y] Values in the same row with different superscripts tend to differ ($0.05 < P \leq 0.10$).

Oleic acid presented a tendency for increase in the HSC group compared to CON ($P = 0.074$), while arachidonic acid was present in significantly higher concentration in HSC group ($P < 0.05$). Milk oxidative stability was not influenced by hempseed cake inclusion in the diet of HSC group, at any time point ($P > 0.05$) (Table 6).

Table 6. Effect of hemp cake supplementation on milk oxidation stability

MDA	Treatment			
(ng/mL)	**CON**	**HSC**	**SEM[1]**	***P-value***
Day 1	2.596	2.512	1.409	0.978
Day 3	5.047	3.594	1.573	0.666
Day 5	10.237	9.002	1.340	0.382

[1]SEM: Standard Error of Mean.

Blood serum parameters

Hemp cake dietary inclusion did not have any significant effect on the level of serum total protein, CPK, Bilirubin, γ-GT, AST, BHBA and LDH of the animals (Table 7). On the other hand, the level of serum urea increased significantly in the serum of HSC cows compared to CON ($P < 0.05$). Moreover, serum Triglycerides tended to be lower for the group HSC ($P = 0.095$). Finally, NEFA concertation was significantly lower in the serum samples corresponding to HSC group ($P < 0.05$).

Table 7. Effect of hemp cake supplementation on serum indices

	Treatment			
Serum parameters	**Control**	**Hemp cake**	**SEM[1]**	***P-value***
Urea (mMol/L)	28.40^{b}	35.80^{a}	1.636	0.012
Protein (g/L)	9.04	8.90	0.162	0.693
AST (U/L)	95.20	81.60	6.491	0.323
γ-GT (U/L)	33.20	35.00	2.233	0.711
CPK (U/L)	276.40	253.60	45.679	0.819
Bilirubin (mg/dL)	0.394	0.294	0.041	0.254
Triglycerides (mg/dL)	13.80^{x}	11.20^{y}	0.778	0.095
LDH (U/L)	1147.20	1014.40	43.078	0.129
BHBA (mMol/L)	712.20	710.20	58.422	0.988
NEFA (mMol/L)	0.158^{a}	0.114^{b}	0.011	0.039

[1]SEM: Standard Error of Mean. ^{ab}Different letters denote significant ($P \leq 0.05$) differences between the dietary treatments. ^{x,y} Values in the same row with different superscripts tend to differ ($0.05 < P \leq 0.10$).

Hempseed cake THC and CBD levels, terpenes profile, and hemp-by products fatty acid composition

The analysis aiming to estimate THC and CBD in both milk and feed samples found the absence of both substances in the experimental samples. More specifically THC peak is expected to be detected at 13.5 min and CBD at 12.01 min, but in the chromatograms no peaks were detected (Figure 1).

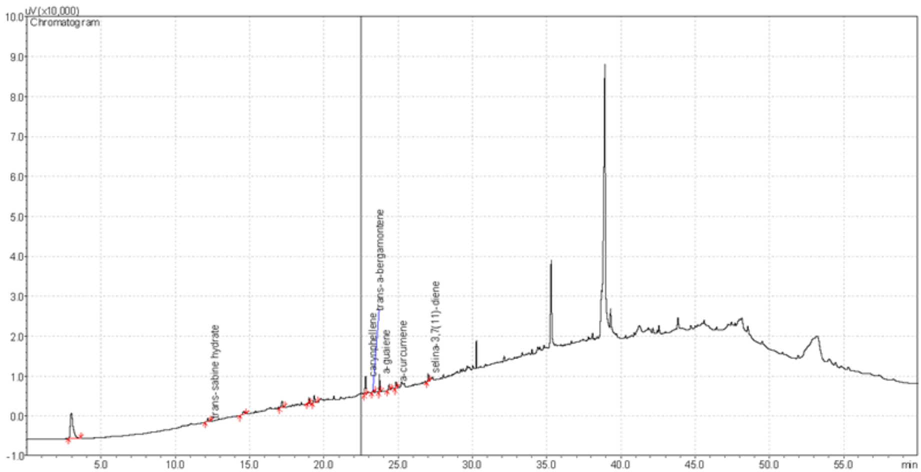

Figure 1. TCH and CBD peaks

This could probably be attributed to the low amount of hempseed cake offered to the cows (3.5 % of the total DM) resulting in undetectable concentrations excreted in the produced milk. Detailed terpenes profiles in hempseed cake samples are presented in Table 8 and hemp by-products fatty acid profile in Table 9.

Table 8. The profile of terpenes found in hempseed cake and the peak time(sec) in GC.

Terpene name	Time of Peak (Seconds)
Hexanal	8.57
Hexene-1-ol	10.05
2-Heptanone	10.89
α-Thujene	12.56
α-Pinene + unknown	12.86
Camphene	13.27
6-Methyl-5- hepten-2-one	13.69
β-Pinene	14.09
β-Myrcene	14.27
δ-3-Carene	15.09
α-Terpinene	15.20
Cymene	15.29
d-Limonene	15.60
γ-Terpinene	16.42
trans-Sabinene hydrate	16.60
cis-Linalool oxide	16.72
Linalool	17.43
d-Fenchyl alcohol	18.04
trans-Pinocarveol	18.82
Borneol L	19.59
1,8-Methandien4-ol	19.81
p-Cymen-8-o	19.81
Terpinene-4-ol	19.92
α-Terpineol	20.22
Piperitenone	24.20
Piperitenone oxide	24.76
α-Ylangene	25.85
α-Copaene	25.97
γ-Caryophyllene	26.76
α-Santalene	27.01

Table 8. Continued

Terpene name	Time of Peak (Seconds)
Caryophyllene	27.16
trans-αBergamotene	27.36
α-Guaiene	27.49
trans-β-Farnesene	27.56
Humulene	27.98
Alloaromadendrene	28.17
α-Curcumene	28.25
β-Selinene	28.75
α-Selinene	28.97
β-Bisobolene	28.97
α-Bulnesene	29.13
Selina-3,7(11)- diene	30.12
Caryophyllene oxide	30.94
Humulene oxide	31.50
Caryophylla-3, 8(13)-dien-5-ol	32.48

Table 9. Hemp by-products fatty acid profile

Fatty acids (%)	Rt (min)	Hemp seed	Hemp Cake	Hemp Hay
C4:0 (Butryic)	5.173			0.184
C6:0 (Caproic)	10.217	0.052	0.352	0.116
C8:0 (Caprylic)	15.682	0.009		
C10:0 (Capric)	20.690	0.002	0.254	
C11:0 (Undecanoic)	22.997			
C12:0 (Lauric)	25.198	0.005		0.094
C13:0 (Tridecanoic)	27.270	0.004	0.108	0.084
C14:0 (Myristic)	29.265	0.068	1.496	1.119
C14:1 (Myristoleic)	30.532			
C15:0 (Pentadecanoic)	31.142	0.038	0.604	0.311
C15:1 (cis-10-Pentadecenoic)	32.377			
C16:0 (Palmitic)	32.980	9.691	36.362	15.048
C16:1 (Palmitoleic)	33.905	0.136	0.251	0.189
C17:0 (Heptadecanoic)	34.675	0.067	0.735	0.314
C17:1 (cis-10-Heptadecenoic)	35.595	0.099	0.209	0.395
C18:0 (Stearic)	36.358	2.477	1.572	4.248
C18:1n9t (Elaidic)	36.887	0.028	0.087	
C18:1n9c (Oleic)	37.102	13.869	5.100	11.782
C18:2n6t (Linolelaidic)	37.920	0.408	0.044	
C18:2n6c (Linoleic)	38.380	55.628	33.915	59.058
C18:3n6 (γ-Linolenic)	39.303	2.822		0.135

Table 9. Continued

Fatty acids (%)	**Rt (min)**	**Hemp seed**	**Hemp Cake**	**Hemp Hay**
C20:0 (Arachidic)	39.475	0.824	0.265	0.792
C18:3n3 (α-Linolenic)	39.892	12.210	16.555	3.414
C20:1n9c (cis-11-Eicosenoic)	40.138	0.438	0.232	
C21:0 (Henicosanoic)	40.922	0.661	0.088	0.307
C20:2 (cis-11,14-Eicossadienoic)	41.358	0.061	0.075	0.077
C20:3n3 (cis-11,14,17-Eicosatrienoic)	42.197			
C22:0 (Behenic)	42.363	0.308	0.552	0.818
C20:3n6 (cis-8, 11, 14-Eicosatrienoic)	42.788	0.011	0.482	0.467
C20:4n6 (Arachidonic)	42.818			0.159
C22:1n9 (Erucic)	42.992	0.020		
C23:0 (Tricosanoic)	43.707	0.031	0.130	0.265
C22:2 (cis-13,16-Docosadienoic)	44.135		0.047	0.137
C20:5n3 (cis-5,8,11,14,17-Eicosapentaenoic)	44.285			
C24:0 (Lignoceric)	45.050	0.010	0.414	0.383
C24:1n9 (Nervonic)	45.653	0.016		
C22:6n3 (cis-4,7,10,13,16,19-Docosahexaenoic)	47.595	0.009	0.074	0.106

DISCUSSION

Animal and agriculture production is gaining significant importance as word population is growing. To secure public food demands, scientists and stakeholders, focus on the maximization of agricultural productivity (Velten et al., 2015). For this purpose, the regenerative system of circular economy becomes progressively the central element of most farming or agricultural investments. The use of agricultural by-products in animal nutrition is one of the most prominent solutions for reducing the production cost of animal products. The industrial hemp cultivation becomes more and more popular not only due to the escalating preference of consumers for hemp nutritional products like flour and oil, but also for its high profitability for the farmers, in contrast to other crops like cotton and corn (Vonapartis et al., 2015).

The results arising from the evaluation of hemp by-products chemical composition in the current study showed that HSC has the highest CP values among the three by-products, however hemp second quality seeds had the highest CF values and hemp hay the lowest. All three by-products had rich content in crude fiber and NDF and ADF. According to the available literature HSC is an promising raw material for ruminant nutrition, containing approximately 340 g/kg DM crude protein, 120 g/kg DM CF and 430 g/kg DM NDF (Bailoni et al., 2021) and is comparable to sunflower, cottonseed meal and legumes. It is speculated that variations in chemical composition emerge from the adoption of different production processes during raw material handling, for oil extraction or flour production. HSC is considered to be an important protein source, capable of replacing a part of commonly used protein sources like soymeal. The inclusion of these feeds in ruminant rations varies from 15 – 30%. A very important note about hemp cake protein is its high digestibility and high percentage of rumen undegradable protein. Crude protein in situ degradability was previously determined in two rumen fistulated cows for different protein sources (hemp, canola, and heated canola meals) and according to the outcomes of that experimental trial, hemp meal is an excellent source of rumen undegradable protein (174 g/kg of CP) (Mustafa et al., 1999). The amino acids profile hasn't been described extensively yet. The main protein found in hemp genus plants is edestine, which is rich in valuable amino acids like glutamic acid and aspartic acid (Wang et al. 2008). Hemp seed protein is rich in sulphur-containing amino acids, methionine and cystine (Odani et al. 1998) and has higher levels of arginine and glutamic acid than soybean meal (Callaway, 2004).

Substituting a portion of cottonseed and soybean meal by HSC, at the level of 3.5 % of the total DM per animal, resulted in similar performance among the control and treatment cows in terms of milk yield, throughout the entire experimental period. In our trial both diets were kept equivalent for their CP and net energy (UFL) content, thus we can assume that HSC nutritional value was adequate to maintain current milk production yields. In the study of Karlsson et al. (2010) lactating cows were offered increasing amounts of HSC (344 g/kg DM CP, 124 g/kg DM CF), at four levels from 0 to 318 g/kg DM, the results showed that HSC inclusion in their diet led to increase on milk yield, only at the level of 143 g/kg DM and had no effect at higher levels. In our study we incorporated lower amounts of HSC in the experimental diet, thus we assume that this could be the reason for the absent enhancing effects. Furthermore, there are nutritional differences among the HSC included in the present study compared to the literature, due to the differences in the production processes.

In the present study HSC inclusion in dairy cows' diet did not affect milk composition. Both diets were formulated to have equivalent nutritional values in contrast to Karlson et al. where they noticed significant differences in milk protein and fat content after offering diets with various HSC inclusion levels, without maintain fixed nutrient compositions (Karlsson et al., 2010). An experiment of Cremonesi et al. (2018) evaluated the effects of linseed and hemp seed included in a percentage of 9.3 % DM in the diet of lactating goats. The results showed that the milk yield had no significant difference between the two groups, but linseed and hemp seed inclusion significantly increased the milk fat content. No differences were detected in milk protein, lactose and urea concentration. Additionally, the total protein content of hemp seed is similar or higher than the most commonly used cereals like corn, wheat and barley, something very important, considering the fact that its price is lower than cereals, so a part of them could be replaced not only with positive impact in feed cost reduction but also in animal product quality.

Somatic cells counts (SCC) was the only parameter that differ among the groups regarding milk characteristics. SCC are used as an indicator of udder health and milk quality. Elevated SCC levels in milk may be indicative of an infection or inflammation in the mammary gland (Harmon, 1994). Our results indicate that HSC inclusion could be associated with SCC mitigation. There is limited research available on the direct impact of hemp inclusion in cow diets on SCC in milk. However, in an article investigating the effects of hemp by-products on milk parameters, authors concluded that hemp bioactive compounds can lower SCC due to hemp's regulatory effect on blood interleukin-1 beta levels, associated with inflammatory processes in the animal organism (Wang et al., 2023). Hemp contains antinutritional components that need to be considered when used as animal feed. The presence of tannins, trypsin inhibitors, phytic acid and saponins in hemp products may reduce the protein availability and the absorption of some minerals and vitamins (Russo and Reggiani, 2015). Russo et al. (2013) reported that the levels of trypsin inhibitors and phytic acid depends on the variety of the plant and despite their high levels in some plants, the 20% participation of hemp by-products in concentrate animal feed is considered safe, especially for ruminants. The levels of congested tannins are non-toxic (< 1% of dry weight) and not so high as to reduce the palatability of the mixture (Russo and Reggiani, 2015). Notwithstanding, hemp antinutritional factors might have an effect primary on monogastric livestock animals that luck enzymes, acting as coping mechanisms such as phytase (Russo and Reggiani, 2015).

Various nutritional approaches are investigated, aiming to enhance dairy products nutritive value, according to consumers demands. The addition of oilseeds, rich in PUFA, in livestock nutrition is the main choice in order to modify milk fatty acid profile, beneficially for human health (Kennelly 1996; Dokou et al., 2023). Caroprese et al. (2012, 2010) reported in a series of experimental trials demonstrating that the addition of PUFA-rich raw materials in the diet of lactating cows and ewes improved milk fatty acid profile. For example, dairy cows diet supplementation with flaxseed, altered milk composition and PUFA levels were higher in flaxseed group compared to the control group (Kennelly, 1996).

In our research the addition of HSC in dairy cow nutrition improved milk fatty acid profile. According to our findings HSC is rich in palmitic acid but also in PUFA such as linoleic and α-linolenic fatty acids. Milk fatty acid analysis revealed that the HSC inclusion led to the production of milk with higher PUFA content (arachidonic acid), in contrast to the basal diet. We can assume that this increase is attributed to the higher availability of PUFA in the diet. This outcome is in agreement with the results reported by Mierlita et al. (2018). Similar also results have been reported in beef cattle where full fat hemp seed dietary inclusion led to increased levels of conjugated linoleic acid and n-3 fatty acids in the carcass, without any negative effect on performance (Gibb et al., 2005) and also in non-ruminant species, hemp seed inclusion in layers increased egg n-3 fatty acid concentrations (Gakhar et al., 2012).

Milk fat content is susceptible to oxidation, a process that can adversely affect its value. Concurrently, higher PUFA concentration in milk may increase the danger of oxidation, resulting from the higher vulnerability of these fatty acids to oxidation (Jacobsen, 2015). In our study we did not observe any difference in milk oxidation status when offering cows HSC, in spite of higher milk PUFA concertation. Hemp oil has been reported to act as a natural antioxidant due to various bioactive molecules (Mierliţă et al., 2018), thus in our experiment we hypothesized that these natural antioxidants present in hemp by-products have a possible protective action against PUFA oxidation.

In the present trial HC inclusion in the diet of dairy cows led to significant differences on serum biochemical parameters. The increase of serum urea observed in the HC group indicates that increased rumen degradable protein was available in the rumen (Bach et al., 2005). Additionally, milk yield and milk protein yield did not differ among the groups implying that animals were provided with adequate rumen undegradable protein, thus we can assume that by providing HSC cows with additional energy, higher milk yield values may be achieved (Hall and Huntington, 2008). NEFA concentration in blood samples represents a biomarker to evaluate cows' energy status. NEFA increase indicates negative energy balance that can result in severe metabolic disorders (Adewuyi et al., 2005). In our study hempseed cake dietary inclusion led to a decrease of serum NEFA concentration providing an insight in cows' supported metabolic status.

In our research it was noticed that milk samples of the hemp cake group were enriched with some special terpenes like caryophyllene and a-guaiene. Caryophyllene is the main hemp terpene but is also found in other common plants like rosemary (*Rosmarinus Officinalis*), cinnamon (*Cynamomum Zylanicum*) and clove (*Sygygium Aromaticum*) and a-guaiene is found in guaiac wood (*Bulnesia Sarmientoi)*. Those plants are commonly used in human nutrition as flavoring additives due to their spicy aroma (Alma et al., 2007; Ormeño et al., 2008). Milk could be enriched by those terpenes to have a special taste and aroma, along with the positive increase of the unsaturated fatty acids. This milk should be further investigated whether it can sustain those special characteristics and considered as superior milk for human health.

CONCLUSION

The addition of hempseed cake could adequately replace a portion of soybean meal and cottonseed without negatively influencing the performance of dairy cows and milk composition. The inclusion of 1 kg hempseed cake per cow daily had beneficial effects on milk fatty acid profile by increasing total PUFA and preserving milk oxidation status indices. The current study also suggested that hemp by-products originating from Greek cultivations can be used as protein source, due to their high nutritional value, their low cost and the large quantity also ensuring an extra income for Greek farmers. Future research efforts should focus on optimizing hempseed cake level of inclusion in dairy cows' nutrition, leading towards elevated animal yields and also the production of final products with higher nutritional value.

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