Animal Science Papers and Reports vol. 42 (2024) no. 1, 81-108
DOI: 10.2478/aspr-2023-0024
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of the Polish Academy of Sciences, Jastrzębiec, Poland

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Effects of *Cannabis sativa* extract on growth performance, meat physicochemical properties, and oxidative status in chickens challenged with *Clostridium perfringens* and lipopolysaccharide*

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(Accepted March 8, 2024)

* This work was supported by the National Science Centre, Grant no. 2018/29/B/NZ9/01351"Bioactivity of cannabidiol and nano-selenium in the maintenance of gut immunological and integrity potential in chickens"
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The present study aimed to evaluate the effect of a Cannabis sativa extract rich in cannabidiol in the diet of chickens under induced stress conditions. This was achieved through the administration of subclinical doses of inflammation-inducing *Clostridium perfringens* bacteria and lipopolysaccharide, which are commonly found in poultry production. A total of 204 male Ross (line 308) chicks were divided into 6 groups, with variations in *Cannabis sativa* extract addition (30 g/1000 g of feed on top) and stress induction methods. At 21 and 22 days of age the birds from the CH1 group were infected (*per os*) with 1 mL of inoculum (brain-heart infusion medium) containing approximately 10^8 CFU/mL of *C. perfringens*. At the same times birds of group CH2 were *per os* administered lipopolysaccharide in a dose of 1 mL containing 250 µg/kg body weight of. The results indicate that incorporating a 30 g/1000 g *Cannabis sativa* extract (CBD) additive in chicken diets leads to an increase in ultimate body weight and maintains weight under stressed conditions ($P<0.05$), without adverse effects on slaughter analysis. Moreover, CBD supplementation does not significantly affect ($P>0.05$) the physical and chemical parameters or primary composition of breast muscle and liver. However, it may alter the n-6/n-3 acid ratio. Additionally, CBD supplementation helps maintain blood biochemical and antioxidant parameters, supporting overall chicken body homeostasis stressed. Incorporating *Cannabis sativa* extracts at 30 g/1000 g of feed presents a potential protective measure to enhance poultry farming in challenging intensive production conditions.

KEYWORDS: cannabidiol / chicken / growth performance / meat quality / inflammation

Poultry production is predominantly based on intensive rearing (95%), which unfortunately reduces the birds' resistance to stress, leading to health issues and potential contamination of meat products [Dal Bosco *et al.* 2021]. To mitigate these effects, alternatives to pharmaceutical agents are being explored. One such alternative is cannabidiol (CBD), derived from hemp (*Cannabis sativa*), which has shown potential for regulating both brain and immune functions [Yekhtin *et al.* 2022, Konieczka *et a*l. 2022]. While CBD's therapeutic effects have been extensively studied in human medicine, its impact on chickens remains unknown due to dosage uncertainties [Klein and Cabral 2006, Izzo and Sharkey 2010, Konieczka *et al*. 2020]. Recent studies have found a correlation between the hosts' intestinal response and meat quality in chickens, with CBD diets reducing bacterial activity in meat [Konieczka *et al*. 2022] and affecting lipid metabolism [Zhou *et al*. 2016].

Necrotizing enterocolitis (NE), caused by the anaerobic bacterium *Clostridium perfringens*, leads to economic losses in the poultry sector due to reduced bird immunity and increased morbidity and mortality. Despite numerous strategies to mitigate *C. perfringens* adverse effects, results remain inconclusive due to limited understanding of its pathogenetic mechanisms [Olkowski *et al.* 2006, Keyburn *et al*. 2010, Koutoulis *et al*. 2015, Keerqin *et al.* 2017]. Some *C. perfringens* strains produce enterotoxins, causing diarrhoea and acute abdominal cramps in humans, making it a common food poisoning agent [Brynestad and Granum 2002, Huang *et al*. 2018, Lahti *et al.* 2008, Lindström *et al*. 2011]. Thus, solutions to prevent *C. perfringens* multiplication in chickens are needed. Another issue is lipopolysaccharide (LPS), a bacterial endotoxin from Gram-negative bacteria. Chickens, resistant to LPS compared to other species, may still exhibit clinical signs and sickness behaviours from *i.v.* LPS administration, negatively impacting performance [Cheng *et al*. 2004, de Boever *et al*. 2009, Warren *et al*. 2010, Liu *et al*. 2015].

The aim of the present study was to determine whether the use of *Cannabis sativa* extract rich in cannabidiol in the diet of chickens under induced stress conditions (through the *in vivo* administration of *Clostridium perfringens* bacteria and LPS at subclinical doses) improves the growth performance, slaughter analysis, chemical composition, physicochemical parameters, fatty acid profile, and oxidative status of selected tissues from slaughtered chickens.

Material and methods

Chemical composition of *Cannabis sativa* extract (CBD)

Hemp panicles (*Cannabis sativa*) obtained from plants were harvested in 2019 at the Institute of Natural Fibres & Medicinal Plants, Poznań, Poland. The plant material was collected, cut, and dried at room temperature. Hemp supercritical carbon dioxide extract was obtained from the Supercritical Extraction Plant, Institute of New Chemical Synthesis, Puławy, Poland. The parameters of the extraction were as follows: pressure, 250 bar; temperature, 60°C; and a flow rate of 40 kg CO_2/1 kg of spent hemp. After the evaporation process, the hemp extract contained 12% cannabidiol, 0.49% tetrahydrocannabinol and 0.38% tetrahydrocannabinolic acid, as determined by HPLC. (Institute of Natural Fibers and Medicinal Plants, Poznań, Poland).

Animals and diets

The experiment was carried out with 204 male Ross 308 chicken broilers randomly allocated to six experimental groups – 34 birds per group. The broilers were reared under standard conditions, and were kept on litter in pens for 35 days. They had free access to water, and were kept under a controlled light cycle [Aviagen 2019]. Chickens were fed diets similar to the standard commercial starter (days 1-7) and

Table 1. Experimental diet

Component (g/kg diet)	Starter 1-7 d.	Grower 8-35 d.	Calculated nutrient density	Starter 1-7 d.	Grower 8-35 d.
Maize	200.0	200.0	ME (kcal/kg)	2900	3000
Soybean meal	306.9	270.0	Crude protein (g/kg)	22.0	20.5
Wheat	432.0	463.0	Crude fiber (g/kg)	2.72	2.7
Lard	20.1	31.5	Crude fat (g/kg)	4.0	5.2
Ronozyme[1]	0.2	0.1	Crude ash (g/kg)	2.7	2.5
Salt	3.3	3.1	Lysine (g/kg)	1.4	1.2
Limestone	15.4	14.0	Methionine (g/kg)	0.6	0.6
Monocalcium phosphate	10.8	8.6	Met. + Cys (g/kg)	1.0	0.9
Choline chloride	1.0	1.0	Threonine (g/kg)	0.9	0.8
DL-Methionine	3.0	2.6	Calcium (g/kg)	0.9	0.9
L-Lysine	3.7	2.8	Available phosphorus (g/kg)	0.4	0.4
L-Threonine	1.1	0.8			
Vitamins + trace minerals[2]	2.5	2.5			

The composition of basal diets (g/kg as-is, unless indicated otherwise) fed to broilers over a 35-day feeding period. [1] Ronozyme WX (Novozymes, Copenhagen, Denmark); 360 FXU/kg diet. [2] Provided IU per kg of feed: vitamin A, 10,000; vit. D3, 4500; mg: vit. E, 80; vit. B1, 1.5; vit. B2, 5; biotin, 0.12; vit. B6, 2.5; vit. B12, 0.02; vit. K, 33; nicotinic acid, 50; folic acid, 1.1; pantothenic acid, 14; choline, 200; betaine, 160; Mn, 120; Zn, 100; Se, 0.35; Cu, 20; Fe, 40; J, 3; and Ca, 0.6.

grower (days 8-35) diets. The same basal diet was used for each treatment, formulated to meet the nutritional requirements of Ross 308 broilers according to their age (as shown in Tab. 1).

Applied Experimental Challenges: Induced Stress Conditions

Experimental groups:
– CON (control treatment);
– CON + CBD (CON with supplementation of CBD extract in the diet (30 g/1000 g diet, on top);
– CH1: *C. perfringens* (as CON but challenged with *C. perfringen*s bacteria);
– CH2: LPS (*Escherichia coli*) (as for CON but with oral gavage of *E. coli* lipopolysaccharide);
– CH1 + CBD: *C. perfringens* + CBD extract;
– CH2 + CBD: LPS (*E. coli*) + CBD extract.

The birds were briefly weighed individually after 4 h of feed deprivation. At 21 and 22 days of age the birds of group CH2 were orally administered LPS (*Escherichia coli*, serotype O55:B5; Sigma Chemical, St. Louis, MO, USA) reconstituted in 0.9% sterile saline (0.5 mg/mL) in a dose of 1 mL containing 250 μg/kg body weight of LPS [Konieczka *et al*. 2019]. At the same times, (day 21 and 22), the birds from the CH1 group were infected (*per os*) with 1 mL of inoculum (brain-heart infusion medium) containing approximately 10^8 CFU/mL of *C. perfringens*, type A, strain 56 bacteria according to a previously validated protocol [Konieczka *et al*. 2020]. The *C. perfringens* bacteria were obtained from infected chickens in Belgium. The strain used was analytically confirmed to be α-toxin- and NetB toxin-positive, and β-toxin- and enterotoxin-negative, as declared by the supplier (Ghent University, Merelbeke, Belgium). The birds in the CON and CON + CBD groups were each administered the same dose of sterile saline and brain-heart infusion medium with a coccidial cocktail (placebo groups) containing the following Eimeria species was administered to all birds at 14 and 15 days of age to create a favourable gut environment for *C. perfringens* colonization.

Sampling procedures

Sixty male chickens were chosen (10 birds from each treatment) for slaughter at the age of 35 days of life that had a body weight similar to the group mean. Tissue samples were collected for slaughter efficiency, chemical composition, physicochemical, biochemical and antioxidant analysis.

Assessment of slaughter efficiency, chemical composition, and the physicochemical properties of pectoral muscles

Upon completion of the feeding experiment, 10 birds (n=10) from each experimental group were randomly selected and weighed prior to slaughter. After slaughter and the carcasses had been cooled, the slaughter efficiency of the chickens was assessed on the basis of a previous report by Michalczuk *et al*. [2016], which determined the percentages of pectoral muscle, legs, and giblets.

The basic chemical composition was determined for the collected pectoral muscle samples: dry weight, crude fat, crude protein, and ash. The determinations were made using the NIR method [Michalczuk *et al*. 2016]. The physicochemical properties of the pectoral muscles were then analyzed (pH_{24} was determined with a pH-meter, CP-411, Elmetron, Zabrze). To determine drip loss, the pectoral muscles were weighed; then, after 24 h, they were dried and reweighed. Drip loss was determined using the difference in masses. The water absorption coefficient was determined according to the Grau and Hamm method [1953], while the meat's color components were identified using a CR-410 colorimeter.

Type III collagen determination was performed using the Chicken Collagen Type 3 ELISA Kit from Bioassay Technology Laboratory (Cat. No. E0314Ch) according to the protocol provided by the manufacturer.

Fatty acids composition

Total lipids from tissues were extracted following the procedure described by Folch et al. [Folch *et al.* 1957]. The fatty acid profile was determined using a gas chromatograph with FID detector according to PN-EN ISO: 5509, PN-EN ISO: 5508, as previously determined by Ciemniewska-Zytkiewicz *et al*. [2015]. Used Restek-2330 capillary column, 105 m, 0.25 mm ID, 0.2 µm df (90% (bis)cyanopropyl /10% cyanopropyl-phenyl polysiloxane). The initial column temperature was 100°C for 4 min, which was hen incrementally increased to 240°C at 3°C/min. The final temperature was kept to a minimum until the elution of the last chromatographic peak. The FID detector temperature was 300°C. H_2 flow 30 mL/min in FID detector, airflow 350 mL/min in FID detector, N2 flow (make-up) 15 mL/min in FID detector. Single-point detector calibration was used for all the determined fatty acid based on the standard. During calibration, the RF (response factor) was determined for each fatty acid methyl ester. Calibration curve using certified BCR-162R reference material. The basic standard contained 37 fatty acids and had the same or similar composition to the standard (Supelco 37 Component Fame Mix), undissolved or dissolved in hexane (stored according to the manufacturer's instructions). The determination of the fatty acid profile was performed in an accredited laboratory (PCA Accreditation Certificate No. AB 439 Issue No. 18 dated 2 August 2019).

Blood biochemical parameters

Blood biochemical parameters were determined in a veterinary laboratory using methods: ALT (alanine aminotransferase) – modified IFCC, TRIS, no P5P; α-amylaze – CNPG 3 – enzymatic; ALP (alkaline phosphatase) – PNPP with AMP as buffer; AST (aspartate aminotransferase) – modified IFCC, TRIS, no P5P; TP (total protein) – biuret method; TCh (total cholesterol) – enzymatic H_2O_2 production; CK (creatinine kinase) – NAC – activated; GLDH (glutamate dehydrogenase) – triethanolamine buffer; GGTP (gamma-GT, aminotransferase) – IFCC standard; UA (uric acid) – uricase; LDH (lactate dehydrogenase) – lactate-pyruvate; TTG (triglycerides) – enzymatic method; BA (bile acid) – diazo-(J-G) w/blank; InP (inorganic phosphorus)

– phosphomolybdate – UV; K (potassium) – ISE-diluted; Na (sodium) – SE-diluted; Fe (iron) – TPTZ [2,4,6-tri-(2-pirydylo)-5-triazyna]. Samples of blood were collected in sterile tubes without an anticoagulant. To obtain serum, the whole blood was centrifuged at 2.000 g for 10 min at 4°C and then placed in the analyzer racks.

Indicators of health status and antioxidant potential

On the day of slaughter, ten birds (n=10) from each experimental group, were randomly chosen for blood collection post-mortem in the amount of 1.5 mL per bird. Laboratory analysis aimed to also determine the activity of selected enzymes and antioxidant compounds by analyzing fragments of breast muscle and liver tissue weighing 5 g.

Measurements for radical scavenging activity in the analyzed tissues were performed by routine assay procedure [Brand-Williams *et al.* 1995] using a synthetic DPPH radical (1,1-diphenyl-2-picrylhydrazyl). Folin-Ciocâlteu reagent was used as an oxidizing reagent, and all the chemicals were purchased from SIGMA-ALDRICH CHEMIE GMBH (Munich, Germany) at the highest available purity. The glutathione (GSH) concentration in the tissues was determined by means of the OXISRESEARCH BIOXYTECH GSH/GSSG-412™ test (Foster City, CA, USA). Before the analysis, the samples were frozen with the addition of M2VP (1-methyl-2-vinyl-pyridium trifluoromethanesulfonate) at a temperature of -80°C. The released, reduced GSH was determined in accordance with detailed instructions provided by the kit's producer. The absorbance reading (λ412) and the measurement of the reaction kinetics were performed using the Synergy 4 microplate reader (BIOTEK; Winooski, VT, USA). The results were calculated using the Gen5 software (BIOTEK). GSH concentration was expressed in thiol groups (mmol-SH groups).

Ethical statement

All procedures in the present study were evaluated and approved by the Local Animal Care and Ethics Committee in Olsztyn (UWM), Poland (Resolution No. 3/2021), and were performed in accordance with the principles of EU (recommendation 2007/526/CE) and Polish Law on Animal Protection. All procedures in this study complied with the ARRIVE guidelines.

Statistical analysis

The normality of the data was checked using the Shapiro–Wilk test. The homogeneity of group variances was examined by the use of the Levene's test. Effects of CBD, and CH and CBD by CH interaction were analyzed using a two-way ANOVA, followed by a Bonferroni post-hoc test for pairwise comparisons, where appropriate.

Results were considered statistically significant when associated with a probability lower than 5%. Results with a probability lower than 1% were considered highly significant. These computations were performed using the PS IMAGO PRO 9.0 for Windows.

Results and discussion

The results of the chickens rearing are shown in Table 2. No significant differences (P>0.05) were found for the main effects of CBD and CH. Only final body weight differed significantly (P<0.001) for the main effect of CBD. Chickens whose diets were enriched with the CBD supplement had a greater body weight on day 35 of 184 g

Table 2. Main effects and interactions for selected chicken rearing parameters

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
Body weight (g):										
day 1	44.24	44.17	0.157	0.351	44.18	44.25	44.43	0.162	0.405	0.276
day 35	1913^{A}	2097^{B}	26.4	<0.001	2051	1942	2012	31.4	0.086	0.009
FCR (kg kg^{-1})	1.64	1.68	0.021	0.485	1.63	1.68	1.69	0.031	0.387	0.511
Mortality (%)	1.96	0.98	0.025	0.259	1.47	2.94	2.94	0.029	0.091	0.187

SEM – standard error of the mean; CBD – cannabidiol; N – no addition of CBD to feed; Y – addition of 30 g/1000 g CBD to feed; CH – challenge; CON – control group; CH1 – induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 – induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.
$^{aA,\cdots}$Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

Table 3. Main effects and interactions for selected parameters of cockerel slaughter analysis

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
Body weight (g)	1924^{B}	2127^{A}	24.9	<0.001	2074	1973	2030	30.6	0.076	0.006
Carcass weight (g)	1369^{B}	1484^{A}	20.8	<0.001	1446	1392	1440	25.5	0.268	0.009
Dressing percentage (%)	69.87	71.25	0.799	0.230	69.66	70.85	71.16	0.979	0.526	0.489
Breast muscles (g/100 g)	30.53	29.95	0.355	0.256	29.65	30.75	30.32	0.435	0.209	0.060
Leg muscles (g/100 g)	19.72	19.71	0.262	0.968	19.96	19.65	19.55	0.320	0.642	0.496
Liver (g /100 g)	3.09	3.06	0.080	0.794	3.23^{a}	3.15^{ab}	2.86^{b}	0.098	0.029	0.021
Fat (g/100 g)	0.47	0.37	0.051	0.200	0.41	0.36	0.49	0.063	0.306	0.213

SEM – standard error of the mean; CBD – cannabidiol; N – no addition of CBD to feed; Y – addition of 30 g/1000 g CBD to feed; CH – challenge; CON – control group; CH1 – induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 – induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.
$^{aA,\cdots}$Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

relative to CON. There was also a significant (P<0.001) interaction effect for the final body weight. For the CON (NO=1916; YES=2232) and CH1 (NO=1845; YES=2100) groups whose diets were enriched with a 3.0% addition of CBD to the feed, the best results were obtained significantly (P<0.001) in relation to final body weight (Fig. 1a).

The slaughter analysis of cockerels (n=10) revealed a significant interaction effects (P<0.001) for the following parameters: slaughter weight, carcass weight, and proportion of liver in the carcass (Tab. 3). Carcasses obtained from the chickens (CON: NO=1333 g; YES=1560 g; CH1: NO=1329; YES=1455 g) whose diet was enriched with added CBD in the feed were significantly heavier (P<0.001) (Fig. 1b). The livers of the chickens that were maintained under induced stress conditions by administering LPS via the *per os* route were significantly (P≤0.05) smaller (NO=3.08; YES=2.64) in the group where the CBD supplement in feed was used (Fig. 1c).

Significant (P≤0.05) interaction effects were found for the parameters pH_{24} and lightness (L_{24}*) (Tab. 4). The pH_{24} for the pectoral muscles that originated from cockerels maintained under induced stress conditions CH1 and that received CBD supplementation in their feed were significantly (P≤0.05) lower (Fig. 2a). Also, the

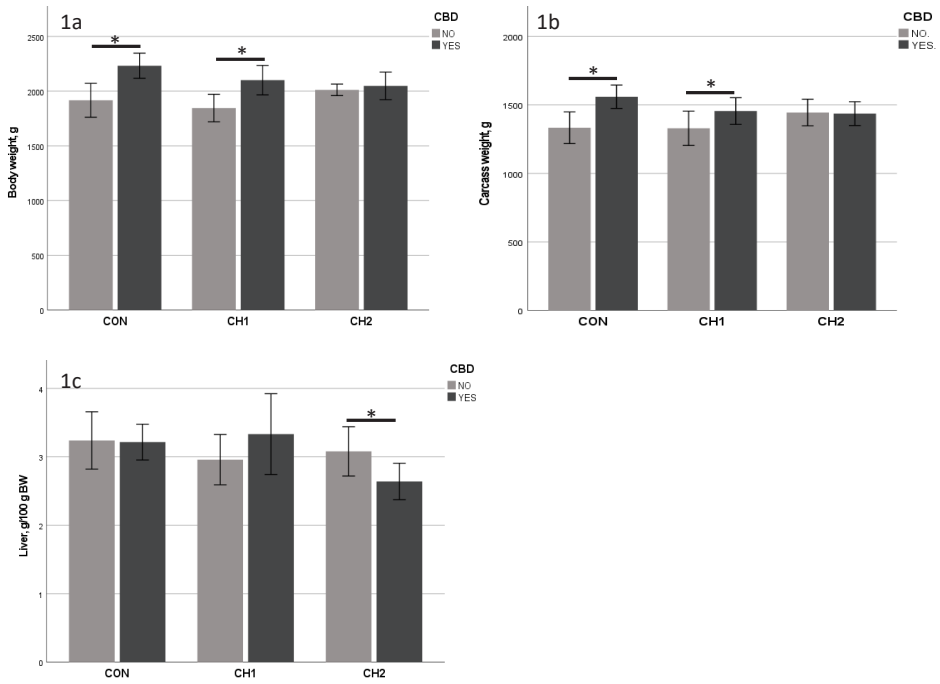

Fig. 1 (a-c). Simple effect of the addition of 30 g/1000 g CBD to feed, or its absence, in tested groups of cockerels for body weight, carcass weight, and liver weight parameters; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os.*

Table 4. Main effects and interactions for selected physicochemical properties in the pectoral muscle

Parameters	CBD				CH				CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
pH_{24}	5.84^{A}	5.93^{B}	0.020	<0.001	5.93	5.86	5.88	0.020	0.079	0.048
Shear force (N)	41.54	38.01	1.330	0.088	41.47	38.25	39.61	1.631	0.382	0.278
L_{24}*	56.7	57.37	0.344	0.067	56.44	56.98	57.68	0.412	0.115	0.019
a_{24}*	15.17	15.19	0.252	0.967	15.54	15.00	15.05	0.311	0.369	0.682
b_{24}*	11.59	12.02	0.320	0.340	11.08	12.2	12.13	0.394	0.088	0.142
Drip loss (%)	0.52^{a}	0.47^{b}	0.017	0.021	0.46^{a}	0.53^{b}	0.50^{ab}	0.025	0.049	0.246

SEM – standard error of the mean; CBD - cannabidiol; N – no addition of CBD to feed; Y – addition of 30 g/1000 g CBD to feed; CH – challenge; CON – control group; CH1 – induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 – induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.

^{aA}...Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

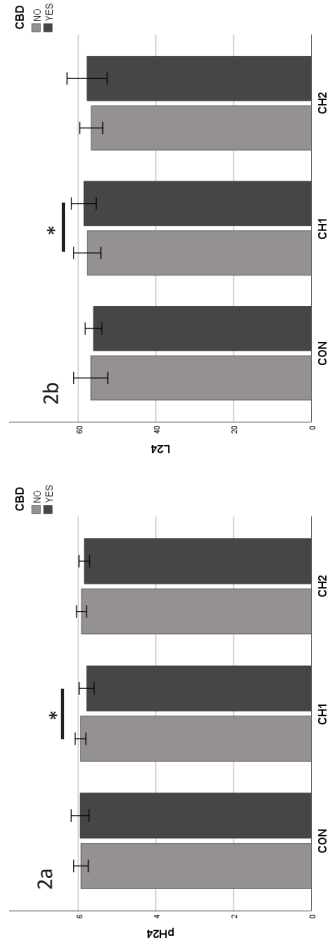

Fig. 2 (a-b). Simple effect of the addition of 30 g/1000 g CBD to feed, or its absence, in tested groups of cockerels for the physico-chemical parameters of the pectoral muscle; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.

pectoral muscles from the CH1 group had significantly (P≤0.01) higher lightness compared to the pectoral muscles of cockerels that were not dosed with *C. perfringens* (Fig. 2b). In addition, significant differences in drip loss were found (P≤0.05) for the main effect of CBD and CH. Breast muscles from cockerels that received the addition of 3.0% CBD in their feed had a 0.05% lower drip loss after 24 h of storage in +4°C refrigeration conditions. When incubated stress conditions were applied, drip loss increased significantly (P≤0.05) compared to CON, where this treatment was not applied (CON=0.46; CH1=0.53; CH2=0.50).

No significant (P>0.05) interaction effects were found for chemical components of breast muscles (Tab. 5). However, significant (P<0.05) main effects for CBD were found for breast muscle content: crude protein, total collagen, moisture content, and crude fat. The breast muscles obtained from cockerels fed a CBD-enriched diet were characterized by lower crude protein (-1.28%) and collagen (-0.30%), but higher fat concentration (+1.06%) compared to the breast muscle from groups not fed a CBD supplement in their feed. For the basic chemical composition of the chickens' livers,

Table 5. Main effects and interactions for selected chemical composition parameters (g/100 g) of breast muscle and liver

Item		CBD				CH				CBD x CH
		N	Y	SEM	P value	CON	CH1	CH2	SEM	P value	P value
Breast muscle	crude protein	23.36^{a}	22.08^{b}	0.421	0.040	22.94	22.23	22.94	0.515	0.422	0.663
	ash	1.68	1.77	0.060	0.319	1.75	1.68	1.75	0.080	0.735	0.895
	collagen	0.64^{A}	0.34^{B}	0.057	0.001	0.50	0.43	0.54	0.070	0.507	0.620
	moisture	74.60^{a}	73.76^{b}	0.240	0.019	74.35	74.15	74.05	0.290	0.763	0.972
	fat content	1.60^{A}	2.66^{B}	0.250	0.006	1.72	2.02	2.65	0.306	0.111	0.551
Liver	crude protein	19.44	19.91	0.200	0.108	19.59	19.94	19.94	0.240	0.422	0.663
	ash	1.31^{a}	1.41^{b}	0.030	0.016	1.38	1.35	1.34	0.030	0.598	<0.001
	collagen	0.71^{A}	0.33^{B}	0.050	<0.001	0.64	0.43	0.48	0.190	0.051	0.001
	moisture	76.47^{A}	75.51^{B}	0.132	<0.001	75.77^{AB}	75.77^{B}	76.43^{C}	0.160	0.013	<0.001
	fat content	2.44	2.79	0.130	0.070	2.89^{A}	2.69^{B}	2.28^{C}	0.160	0.038	<0.001

SEM – standard error of the mean; CBD – cannabidiol; N – no addition of CBD to feed; Y – addition of 30 g/1000 g CBD to feed; CH challenge; CON – control group; CH1 – induced stress using *C. perfringens* at a dosage of 10^{8} CFU *per os*; CH2 – induced stress usin *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.
$^{aA...}$Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

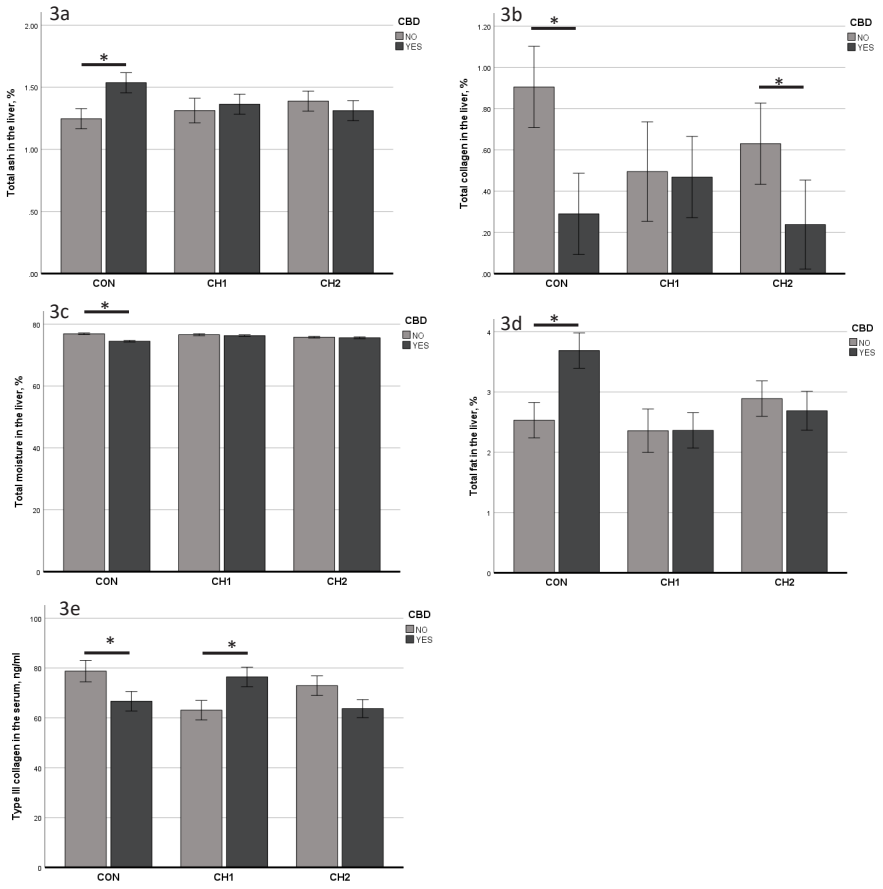

Fig. 3 (a-e). Simple effect of the addition of 30 g/1000 g CBD to feed, or lack thereof, in cockerel groups tested for baseline liver chemistry; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*

significant (P≤0.01) interaction effects were found for crude ash, total collagen, moisture content, and crude fat content. The application of a 3.0% CBD addition to the feed increased (P≤0.01) the concentration of total ash in the livers of cockerels that were not maintained under induced stress conditions. Total ash content in the groups under induced stress conditions did not differ significantly (P>0.05), regardless of whether dietary CBD was used or not (Fig. 3a). The use of CBD in the chickens' diet significantly (P≤0.01) reduced total collagen content in the liver. In the CON group, the addition of 3.0% CBD to the feed reduced collagen concentrations by 0.62%, while in chickens maintained under induced stress conditions through the administration of

LPS, the total liver collagen concentration decreased by 0.53%. No effect (P>0.05) on the total collagen concentration in the livers of the cockerels was found when a subclinical dose of *C. perfringens* was applied (Fig. 3b). The addition of CBD to the feed had a significant (P≤0.01) effect on liver water content (-2.40%). The application of CBD to the groups with subclinical doses, CH1 and CH2, had no significant (P>0.05) effect on liver water content (Fig. 3c). This was similar for liver fat content. The use of induced stress conditions had no significant effect (P>0.05) on liver fat content, regardless of whether a CBD supplement was used or not in the chickens' diet. Only in the CON group did the addition of 3.0% CBD to the feed have a significant (P≤0.01) effect on increasing the fat concentration in the livers of the cockerels by-by 1.16% relative to the group where no CBD addition to the feed was used (Fig. 3d).

The administration of subclinical doses of *C. perfringens* and LPS to chickens significantly (P≤0.01) affected the concentration of type III collagen in the pectoral muscle (Tab. 6). Maintaining chickens under induced stress conditions reduces type III collagen levels by approximately 25% relative to the CON group. No main effect of CBD (P>0.05) was found for the addition of CBD to the feed to alter the concentration of type III collagen in the pectoral muscle. A significant interaction effect was found (P≤0.01) only for serum type III collagen concentration. The addition of CBD decreased serum type III collagen concentration by 15%, while, for the CH1 and CH2 groups, only in the CH1 group did the addition of CBD to the feed significantly (P≤0.05) increase type III collagen content-by 17.5% (Fig. 3e).

Significant interaction effects (P≤0.05) were found in the fatty acid profile for the mammary's fatty acids for C14:0, C15:0, C16:1, C17:0, C18:1 (cis-9), ALA, C22:0, and C20:3. Significant interaction effects (P≤0.05) were also identified for fatty acid content: PUFAs, n-3 and n-6 family acids, and the ratio of n-6 to n-3 fatty acids (Tab. 7). In analyzing essential fatty acids, it was found that the use of CBD supplementation in feed had a significant effect (P≤0.05) on reducing PUFA concentration in the pectoral muscle: a 3% reduction in cockerels exposed to induced stress by through the administration of *C. perfringens per os* (CH1) - Figure 4a. When a subclinical dose of LPS was

Table 6. Main effects and interactions for type III collagen concentration (ng/ml) in the analyzed tissues

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
Breast muscles	282.84	286.08	5.773	0.694	345.51^{A}	252.31^{B}	255.56^{B}	7.061	<0.001	0.224
Liver	251.8	267.26	6.962	0.127	256.62	263.51	258.45	8.520	0.837	0.337
Serum	70.49	67.85	1.970	0.350	72.73	68.33	66.44	2.416	0.199	0.001

SEM - standard error of the mean; CBD – cannabidiol; N – no addition of CBD to feed; Y - addition of 30 g/1000 g CBD to feed; CH - challenge; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.
^{AB}Means bearing different superscripts differ significantly at P≤0.01.

Table 7. Main effects and interactions for the fatty acid profile in pectoral muscles (g/100 g)

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
C14:0	0.47	0.48	0.005	0.788	0.45^{A}	0.50^{B}	0.47^{A}	0.012	<0.001	0.176
C15:0	0.31^{A}	0.20^{B}	0.024	0.001	0.3	0.23	0.23	0.033	0.078	0.010
C16:0	13.71	13.53	0.101	0.188	13.96^{A}	13.65^{AB}	13.26^{B}	0.126	0.001	0.038
C16:1	1.9	1.97	0.076	0.435	2.13^{A}	1.86^{AB}	1.80^{B}	0.084	0.014	0.004
C17:0	0.16	0.16	0.014	0.075	0.16	0.16	0.16	0.018	0.992	0.014
C17:1	0.10	0.07	0.266	0.373	0.11	0.08	0.07	0.027	0.408	0.433
C18:0	5.81	5.79	0.072	0.886	5.85	5.87	5.69	0.099	0.279	0.278
C18:1 (trans-9)	0.08	0.08	0.011	0.714	0.08^{A}	0.08^{A}	0.07^{B}	0.014	<0.001	0.258
C18:1 (cis-9)	33.54	33.45	0.235	0.783	34.24^{A}	33.22^{BC}	33.03^{C}	0.286	0.007	0.042
C20:0	0.34	32.00	0.019	0.059	0.33	0.33	0.32	0.015	0.571	0.562
C18:3, ALA	0.84	0.86	0.011	0.320	0.84	0.85	0.86	0.011	0.474	0.310
C20:1	0.34	0.31	0.013	<0.001	0.34^{A}	0.32^{B}	0.31^{BC}	0.019	0.001	0.225
C20:2	0.58	0.54	0.026	0.213	0.54	0.60	0.54	0.025	0.073	0.186
C22:0	0.34	0.31	0.011	0.161	0.32^{AB}	0.36^{A}	0.30^{B}	0.027	0.027	0.004
C20:3	2.78	3.02	0.115	0.122	3.27^{A}	2.91^{AB}	2.52^{B}	0.133	0.001	0.007
C20:4, AA	0.16	0.15	0.012	0.868	0.17	0.15	0.14	0.012	0.265	0.982
C24:1	1.15	1.07	0.059	0.219	1.14	1.19	1.00	0.064	0.051	0.346
C22:6, DHA	0.18	0.20	0.012	0.133	0.18^{AB}	0.22^{A}	0.17^{B}	0.019	0.017	0.257
SFA	21.08	20.74	0.144	0.089	21.38^{A}	21.00^{A}	20.35^{B}	0.174	<0.001	0.633
MFA	37.03	36.89	0.256	0.699	37.98^{A}	36.76^{B}	36.14^{BC}	0.315	0.001	0.033
PUFA	42.52	42.74	0.268	0.561	42.27^{A}	42.04^{A}	43.58^{B}	0.328	0.003	0.020
PUFA n-3	3.87	4.08	0.128	0.246	4.39^{A}	3.40^{AB}	3.56^{B}	0.159	0.002	0.010
PUFA n-6	38.74	38.62	0.264	0.754	38.01^{A}	37.95^{A}	40.09^{B}	0.326	<0.001	0.007
n-6/n-3 PUFA	9.83^{A}	10.56^{B}	0.261	0.049	9.64	10.33	10.61	0.324	0.094	<0.001

SEM - standard error of the mean; CBD - cannabidiol; N - no addition of CBD to feed; Y - addition of 30 g/1000 g CBD to feed; CH - challenge; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os,* :ALA - α-Linolenic acid; AA - arachidonic acid; DHA - docosahexaenoic acid; PUFA - polyunsaturated fat.

$^{aA,\cdots}$Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

applied (CH2), the addition of CBD to the feed significantly (P≤0.01) increased the concentration of n-3 fatty acids by 21% compared to the group of roosters exposed to LPS but without the addition of 3.0% CBD (Fig. 4b). In the CH1 group, the addition of CBD to the feed significantly (P≤0.01) reduced the content of n-6 family acids by 4% (Fig. 4c). The use of a 3.0% CBD addition to feed significantly (P≤0.01) affected the n-6/n-3 ratio (Fig. 4d). The breast muscles from cockerels that had been maintained under standard conditions (CON) and had received CBD supplementation in their feed had a 25% higher n-6/n-3 ratio than muscles from cockerels not receiving CBD supplementation in their feed. For the CH1 group, the ratio was 20% higher. Only the breast muscles from cockerels maintained under induced stress conditions (CH2) were characterized by a significantly (P≤0.01) lower n-6/n-3 ratio-by 25%-when CBD was added to the diet.

Significant interaction effects in the fatty acid profile of livers were not found (P>0.05) for C15:0, C17:1, or C18:1 (trans-9) (Tab. 8). The application of a 3.0% CBD supplement to the feed influenced (P≤0.05) a change in the concentration of essential fatty acids (EFAs) in the liver under induced stress conditions. The use of CBD supplementation in the feed influenced the reduction of LA acid by nearly 19% (P≤0.05); while under induced stress (CH2) conditions, CBD supplementation

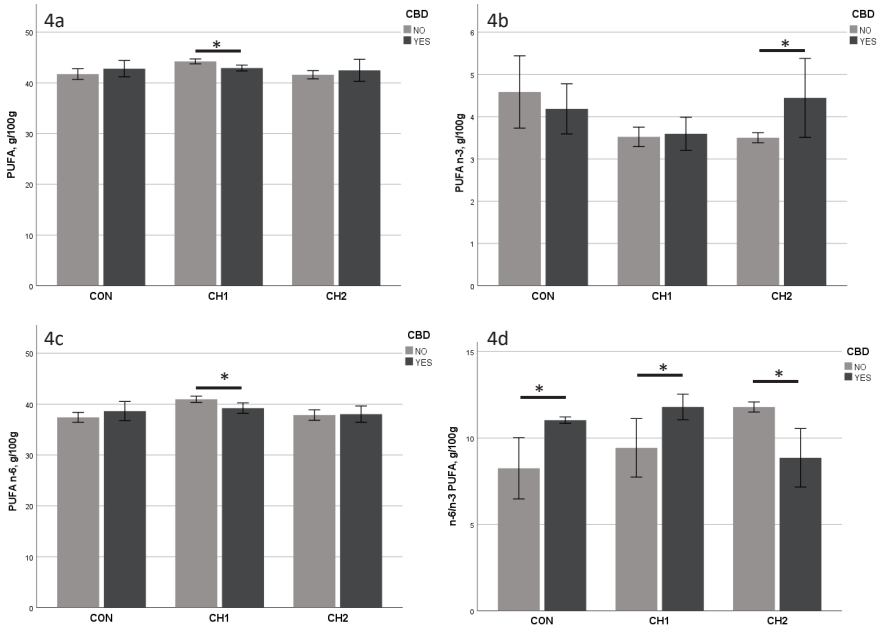

Fig. 4 (a-d). Simple effect of the addition of 30 g/1000 g CBD to feed, or lack thereof, for cockerel groups tested thefor fatty acids profile of the pectoral muscle; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os.*

influenced an increase in LA acid levels in the liver by nearly 7% (Fig. 5a). The presence of GLA (gamma-Linolenic acid) acid was only found in the livers of chickens maintained under induced stress conditions. The application of the CBD supplement alone under standard chick maintenance conditions did not affect (P>0.05) the levels of GLA acid in the livers of cockerels (trace value means ≤0.050 g/100 g). The application of subclinical doses *per os* (CH1 and CH2) influenced the presence of GLA acid, but only in the CH2 group did it significantly (P≤0.01) increase the concentration of this fatty acid: almost 59% relative to the CH2 group in which no CBD was added (Fig. 5b). The addition of CBD to the feed had a significant (P≤0.01) effect in reducing (by 47%) AA levels (Fig. 5c), while in the CH1 group, it was by 25%. The same is true for the fatty acid DHA. The use of CBD in the birds' diet significantly (P≤0.05) reduced liver GLA by 41% (CON) and by 23% in the CH1 group. The use of a subclinical dose of LPS (CH2) influenced (P≤0.01) an increase in liver GLA levels (by 43%) when CBD was added to the birds' diet; this was in contrast to the CON and CH1 groups (Fig. 5d). The addition of CBD to the feed significantly (P≤0.01) decreased n-3 family acids in the CON group (by 41%) and in the CH1 group (by 24%), while in the CH2 group it increased PUFA n-3 by 27% relative to the

Table 8. Main effects and interactions for fatty acid profile in the livers (g/100 g)

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
C14:0	0.24^{A}	0.29^{B}	0.115	0.002	0.30^{A}	0.25^{a}	0.25^{b}	0.145	0.015	<0.001
C15:0	0.12^{A}	0.31^{B}	0.301	<0.001	0.13	0.26	0.25	0.037	0.051	0.081
C16:0	13.31^{A}	14.80^{B}	0.216	<0.001	15.48^{A}	13.48^{B}	13.20^{BC}	0.256	<0.001	<0.001
C16:1	0.55^{A}	0.78^{B}	0.051	0.002	0.96^{A}	0.58^{B}	0.47^{BC}	0.078	<0.001	<0.001
C17:0	0.24	0.24	0.015	0.509	0.21^{A}	0.27^{B}	0.24^{C}	0.017	<0.001	<0.001
C17:1	0.01	0.01	0.010	0.741	0.01	0.01	0.01	0.011	0.430	0.106
C18:0	18.67^{a}	17.83^{b}	0.276	0.032	18.59	17.82	18.39	0.330	0.270	0.004
C18:1 (trans-9)	0.11^{a}	0.08^{b}	0.017	0.018	0.13^{A}	0.07^{B}	0.08^{BC}	0.016	0.004	0.418
C18:1 (cis-9)	15.63^{A}	18.97^{B}	0.72	0.003	19.50^{a}	16.85^{ab}	15.55^{b}	0.894	0.013	<0.001
C18:2, LA	24.80^{a}	23.78^{b}	0.304	0.024	21.33^{A}	25.73^{B}	25.82^{BC}	0.389	<0.001	<0.001
C20:0	0.16^{A}	0.11^{B}	0.016	<0.001	0.18^{A}	0.13^{B}	0.10^{B}	0.015	<0.001	<0.001
C18:3, n-3 GLA	0.08^{A}	0.12^{B}	0.015	0.001	trace	0.13^{A}	0.17^{B}	0.014	<0.001	0.001
C18:3, n-6 ALA	0.22	0.18	0.029	0.060	0.18	0.20	0.23	0.026	0.181	0.121
C20:1	0.28	0.29	0.014	0.754	0.26	0.29	0.30	0.026	0.285	0.017
C20:2	1.38^{A}	1.08^{B}	0.045	<0.001	1.09^{A}	1.17^{A}	1.44^{B}	0.042	<0.001	<0.001
C22:0	0.06^{A}	0.03^{B}	0.019	<0.001	0.04	0.06	0.05	0.014	0.085	<0.001
C20:3, n-6	1.32^{A}	1.13^{B}	0.037	<0.001	1.33^{a}	1.17^{b}	1.18^{b}	0.040	0.021	0.039
C20:3, n-3	18.55^{A}	15.79^{B}	0.418	<0.001	16.36	17.24	17.92	0.501	0.108	<0.001
C20:4, AA	0.31^{A}	0.21^{B}	0.025	<0.001	0.30^{A}	0.21^{B}	0.25^{AB}	0.022	0.008	0.034
C24:1	2.52^{A}	2.05^{B}	0.076	<0.001	2.03^{A}	2.35^{AB}	2.49^{B}	0.096	0.005	<0.001
C22:6, DHA	1.07	0.99	0.057	0.312	1.00	1.03	1.07	0.069	0.765	<0.001
SFA	33.20	33.92	0.279	0.070	35.09^{A}	32.85^{B}	32.73^{BC}	0.337	<0.001	0.001
MFA	3.43	3.30	0.065	0.149	3.40	3.28	3.41	0.078	0.434	0.007
PUFA	47.95^{A}	43.76^{B}	0.665	<0.001	42.17^{A}	47.26^{B}	48.14^{BC}	0.819	<0.001	<0.001
PUFA n-3	19.76^{A}	17.01^{B}	0.437	<0.001	17.36	18.57	19.23	0.537	0.055	<0.001
PUFA n-6	27.94^{A}	26.40^{B}	0.319	0.001	24.14^{A}	28.46^{B}	28.91^{BC}	0.384	<0.001	<0.001
n-6/n-3 PUFA	1.50	1.60	0.056	0.413	1.41	1.63	1.55	0.065	0.062	<0.001

SEM - standard error of the mean; CBD - cannabidiol; N - no addition of CBD to feed; Y - addition of 30 g/1000 g CBD to feed; CH - challenge; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os;* LA - linoleic acid; GLA - gamma-linolenic acid; ALA - α-linolenic; AA - arachidonic acid; DHA - docosahexaenoic acid; PUFA - polyunsaturated fat

[aA...]Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

groups without CBD addition (Fig. 5e). For PUFA n-6 content, the addition of 3.0% CBD to the chicken feed was found to have a significant effect (P≤0.05) in decreasing liver PUFA n-6 concentration (by 20%) (CON) and increasing PUFA n-6 (by 6.5%) in the group where LPS-induced stress conditions were applied (Fig. 5f). The ratio of n-6 to n-3 families of acids changed with the addition of CBD to the feed. CBD was found to have significantly (P≤0.05) increased the n-6/n-3 ratio in the CON group by 20% and the CH1 group by 21%; while in the LPS-induced group the ratio decreased by 26% relative to groups maintained under the same environmental conditions but that did not have CBD added to their feed (Fig. 5g).

Blood biochemical parameters are shown in Table 9. Significant interaction effects (P≤0.05) were found for the AST and CK parameters. Maintaining chickens under induced stress conditions by administering *per os* LPS and giving chickens the CBD supplement had a significant (P≤0.05) effect, by increasing AST levels in their blood by 31%. For the CON and CH1 groups, CBD application had no significant (P>0.05) effect on blood AST levels (Fig. 6a). The addition of CBD to feed significantly reduced the level of CK in the blood of chickens in the CON group by 68% and in the CH1

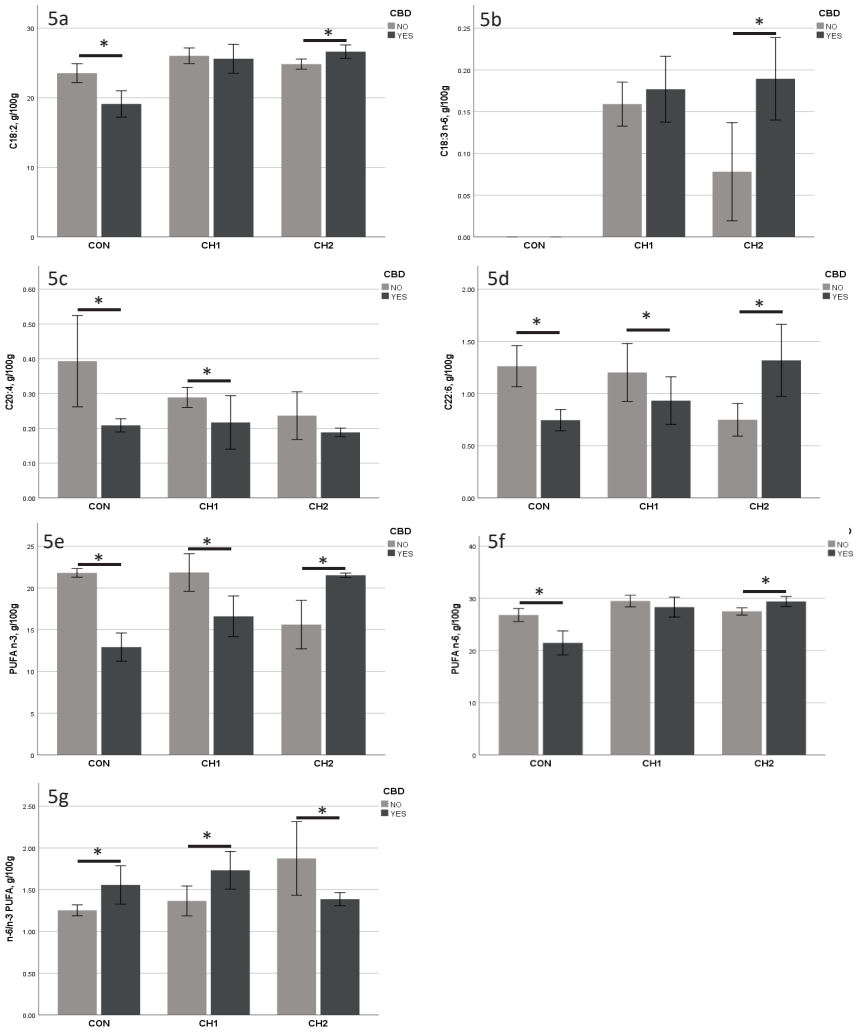

Fig. 5. (a-g). Simple effect of the addition of 30 g/1000 g CBD to feed, or lack thereof, for the cockerel groups tested for liver fatty acid profile; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU per os; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. per os.

group by 58%, while in the CH2 group, the addition of CBD to feed had no significant effect (P>0.05) on the reduction of the CK parameter (Fig. 5b). The main effect of CBD was found to be significant (P≤0.05) for an increase in the parameters TP, TCh, BA, and Na; while the addition of CBD to the feed significantly (P≤0.05) decreased the levels of CK, GLDH, InP, and K.

Table 9. Main effects and interactions for selected assays in the cockerels' blood

Parameters	CBD				CH					CBD x CH
	NO	YES	SEM	P value	CON	CH1	CH2	SEM	P value	P value
ALT (U/L)	1.88	2.08	0.184	0.457	1.85	2.00	2.09	0.229	0.744	0.724
αamylase (U/L)	590.51	637.66	30.755	0.287	580.88	586.05	675.33	37.654	0.155	0.083
ALP (U/L)	4874	3916	433.3	0.129	4654	4025	4506	530.7	0.684	0.634
AST (U/L)	396.84	407.57	25.251	0.766	353.35	414.54	438.73	30.939	0.150	0.025
TP (g/dL)	2.84^{A}	3.08^{B}	0.052	0.002	2.96	2.98	2.94	0.064	0.930	0.676
TCh (mg/dL)	109.05^{A}	132.02^{B}	3.121	<0.001	125.16^{a}	125.58^{a}	110.86^{b}	3.821	0.015	0.305
CK (U/L)	31665^{A}	17836^{B}	2385.1	<0.001	23505	21737	29010	2921.2	0.202	<0.001
GDLH (U/L)	6.12	5.84	0.489	0.678	5.66	6.20	6.08	0.592	0.794	0.339
GGTP (U/L)	23.22	24.24	1.144	0.533	23.48	21.54	26.16	1.446	0.081	0.659
UA (mg/dL)	5.79	5.28	0.365	0.322	6.45^{A}	4.02^{B}	6.13^{A}	0.441	0.001	0.731
LDH (U/L)	4382^{a}	3092^{b}	343.512	0.013	3344	3638	4229	420.7	0.331	0.925
TTG (mg/dL)	62.72	70.36	3.779	0.162	87.65^{A}	55.59^{B}	56.39^{B}	4.626	<0.001	0.030
BA (mg/dL)	19.09^{A}	24.85^{B}	1.931	0.043	22.60	18.74	24.56	2.364	0.223	0.649
InP (mg/dL)	9.61^{A}	8.73^{B}	0.150	<0.001	9.07	9.39	9.05	0.192	0.375	0.152
K (mg/dL)	38.48^{a}	35.06^{b}	1.011	0.023	36.36	35.85	38.09	1.249	0.417	0.055
Na (mg/dL)	367.21^{a}	373.43^{b}	1.796	0.020	362.80^{A}	380.42^{B}	367.74^{A}	2.197	<0.001	0.131
Ca (mg/dL)	11.42	11.46	0.134	0.847	11.47	11.48	11.36	0.164	0.829	0.225
Fe (mg/dL)	80.95	78.29	2.012	0.356	83.58^{A}	71.48^{A}	83.80^{B}	2.469	0.001	0.494

SEM - standard error of the mean; CBD - cannabidiol; N - no addition of CBD to feed; Y - addition of 30 g/1000 g CBD to feed; CH - challenge; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*; ALT (alanine aminotransferase); ALP (alkaline phosphatase); AST (aspartate aminotransferase); TP (total protein); TCh (total cholesterol); CK (creatinine kinase); GLDH (glutamate dehydrogenase); GGTP (gamma-GT, aminotransferase); UA (uric acid); LDH (lactate dehydrogenase); TTG (triglycerides); BA (bile acid); InP (inorganic phosphorus); K (potassium); Na (sodium); Fe (iron).
$^{aA,\cdots}$Means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

The oxidative status of breast and liver samples (Tab. 10) did not differ significantly (P>0.05) in the conducted study. The results obtained did not deviate from those obtained from current poultry production.

The poultry sector seeks feed additives to enhance bird health and performance. *Cannabis indica*, known as marijuana, has potential medicinal value due to its high Δ9-tetrahydrocannabinol levels [Grotenhermen *et al*. 2017, Rehman *et al.* 2021]. In contrast, *Cannabis sativa*, with its non-psychoactive properties and high CBD content, is of interest as a livestock diet ingredient. The endocannabinoid system, present in both invertebrates and vertebrates [Salzet and Stefano 2002, Oltrabella *et al.* 2017, Breivogel *et al*. 2018] is linked to CBD's action and regulates various physiological processes [Atalay *et al*. 2020, della Rocca and Di Salvo 2020]. Necrotic enteritis (NE) in chickens can potentially be addressed using *Cannabis sativa*-derived CBD in their diet. This could serve as an alternative preventive strategy, reducing antibiotic use and the risk of drug-resistant microorganisms. Chickens can be infected with *C. perfringens* without showing clinical signs, posing a risk of pathogen transfer to the food chain. The non-psychotropic component of hemp seeds, CBD, appears to have significant benefits for humans and animals [Konieczka *et al*. 2020].

In the present study, a challenge agent was used to induce stress in chickens in two ways by administering *per os* doses of subclinical Gram-positive bacteria *Clostridium perfringens* and a *per os* doses of lipopolysaccharide extracted from the cell membrane of *E. coli* bacteria. Toll-like receptor 4 (TLR-4) recognizes LPS from Gram-negative

Table 10. Main effects and interactions for antioxidant parameters in the selected tissues of the cockerels

Item		CBD				CH				CBD x CH
		N	Y	SEM	P value	CON	CH1	CH2	SEM	P value	P value
Breast muscle	DPPH (%)	51.38	52.10	0.387	0.568	52.21	50.81	51.94	0.515	0.250	0.581
	GSH (mmol-SH)	0.09	0.10	0.062	0.895	0.08	0.11	0.12	0.072	0.115	0.380
Liver	DPPH (%)	83.14	84.01	0.079	0.406	84.79	83.85	84.02	0.127	0.441	0.492
	GSH (mmol-SH)	0.72	0.75	0.081	0.357	0.79	0.68	0.71	0.096	0.381	0.716

SEM – standard error of the mean; CBD – cannabidiol; N – no addition of CBD to feed; Y – addition of 30 g/1000 g CBD to feed; CH – challenge; CON – control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 CFU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.

Fig. 6 (a-b). Simple effect of the addition of 30 g/1000 g CBD to feed, or lack thereof, in cockerel groups tested for AST and serum CK; * - mean values marked with this symbol are significantly different, P≤0.05; CON - control group; CH1 - induced stress using *C. perfringens* at a dosage of 10^8 IU *per os*; CH2 - induced stress using *E. coli* lipopolysaccharide (LPS) at a dosage of 250 µg/kg b.w. *per os*.

bacteria and toll-receptor 5 (TLR-5) recognizes bacterial flagellin, a subunit of the bacterial flagellum; while, in addition, bacterial TLR antagonists increase the expression levels of additional pro-inflammatory cytokines such as IL-1βand IL-6 [Csernus *et al*. 2020], which may negatively affect normal chickens growth. NE, induced by *C. perfringens*, causes chronic damage to the intestinal mucosa, which can lead to reduced growth performance in chickens through reduced nutrient absorption due to a less efficient digestive process [Timbermont *et al*. 2011, Prescott *et al*. 2016,

Zahoor *et al*. 2018]. CBD can modulate the immune response, which may provide a first line of defense against infection [Konieczka *et al.* 2020].

In the available literature, there is little or no research on the use of CBD in the diets of chickens and its effect on various parameters,, be it the effect on the meat yield or the health status of the chickens. Studies mostly focus on feeding chickens hemp seeds, which contain small amounts of CBD in their composition but also many other components such as carbohydrates, proteins (arginine, methionine, and cysteine), vitamins (e.g. γ-tocopherol), and fats extracted from the seeds, as well as essential amino acids, fiber, vitamins, and minerals (Fe, Zn, Cu, and Mn), cannabinoids, and terpenes [Yu *et al*. 2005, della Rocca and Di Salvo 2020, Rehman *et al.* 2021, Valizadehderakhshan *et al.* 2021]. To the best of our knowledge, this is one of the first studies to determine the effect of the bioactive properties of CBD in broiler chickens on rearing performance, and breast muscle and liver quality under induced stress conditions. The rearing results of the chickens in the present study confirm that the induction of stress using the *per os* route does not impair rearing performance, in contrast to studies [Chen *et al*. 2018, Gharib-Naseri *et al*. 2019] in which a subclinical dose of *Clostridium perfringens* and LPS was also used. In the current study, the chicken rearing results obtained do not deviate from those obtained from commercially reared broiler chickens. However, the administration of CBD alone, at a dose of 3.0% per kg of feed, improved final body weight, which may be related to improved feed palatability and increased feed intake. Furthermore, the use of hemp seed, which also contains CBD in its composition, but importantly also several other compounds, has been shown to increase the body weight of cockerels [Sevcikova *et al.* 2006]. There are also reports that hemp seed does not negatively affect feed conversion ratio (FCR), which may have an indirect effect on understanding the use of CBD supplementation in chicken feed [Mahmoudi *et al*. 2015]. The use of stress inducers and CBD alone did not increase chicken mortality, confirming, on the one hand, the lack of negative effects of CBD on chicken health and the correctness of the use of a subclinical dose, which, as stated, does not increase bird deaths but may only have a positive or negative effect on the rearing performance of the chickens. The slaughter analysis carried out showed that CBD application under induced stress conditions had no negative effects. Only when 3.0% CBD was administered to the CH2 groups, was a lower proportion of liver weight relative to body weight found in the CON cockerels. The knowledge of changes in liver weight in chickens exposed to LPS states that endotoxin-induced changes have already been repeatedly observed. It is known that changes in liver weight are caused by an increase in its metabolic function [Curtis *et al.* 1980, Xie *et al.* 2000, Mireles *et al.* 2005]. Analyzing similar studies in which chicken organisms were exposed to LPS and, in this case, treatment with berberine, which has properties similar to that of morphine and codeine, were also found to have a lower liver weight relative to body weight in the cockerels, in a similar way to when LPS and CBD were used in the chickens' diet [Shen *et al*. 2010]. Similarly, cannabidiol (CBD) demonstrates synergistic effects with sub-analgesic doses of morphine in an acute

pain model (i.e., acetic acid writhing) but not against thermal pain [Neelakantan *et al.* 2015]. Relative liver weight may be a good indicator of the magnitude of the acute phase response because the liver is the site where acute phase proteins are synthesized [Koj 1996]. Although this was not analyzed in this study, it can be suggested that the addition of 3.0% CBD to feed reduces the negative effects of LPS on chickens, which is undoubtedly linked to metabolic processes in the liver. Studies using other animal species have shown increased liver weight, for example, in rhesus monkeys, among others, and elevated liver enzymes, for instance, in dogs, when CBD was administered at doses as low as 2 mg/kg body weight [Rosenkrantz *et al*. 1981, Gamble *et al*. 2018].

The use of a 3.0% CBD addition to chicken feed has a lowering effect on the pH value of the pectoral muscles in cockerels that are given a subclinical dose of *C. perfringens*. Only, in this case, the results showed a slightly higher rate of post-mortem acidification of the meat. In the experiment conducted on the CH1 group, a slight increase in the L_{24} parameter of the pectoral muscle was found, which contrasts with other similar study results [Yang *et al*. 2010, Zhou *et al*. 2015]. However, slight changes in the lightness of the meat or a general lack of change in the infected meat may be difficult to distinguish for the potential consumer. The addition of CBD alone (30 g/kg feed) does not alter the color parameters, which is a plus if consumers are accustomed to the product.

The analysis of the chemical components of breast and liver showed that significant interactions were only observed within the liver tissue. The results from the study show that a mild infection with *C. perfringens* [Olkowski *et al*. 2008], or induced by LPS from *E. coli*, may not affect changes in the chemical composition of the breast muscle tissue, which could be a potential risk in the supply chain. Only in liver tissue was a significantly higher concentration of ash and lower collagen, water, and fat found after CBD administration to the feed. In the CON where only the CBD addition to the feed was used, an increased proportion of fat fraction was found in the liver, which is associated with the CBD administration itself, but importantly, no change in fat content was found in the CH1 and CH2 groups. The fat levels in the livers from these groups remained the same. The present study did not analyze CBD residues in the pectoral muscle; however, based on our team's study [Konieczka *et al.* 2020], feeding the concentration of CBD in the pectoral muscle after a diet of 15 g/1000 g (CBD) for 35 days was 141.54±95.54 ng/g (on a dry weight basis). The overall liver collagen concentration depended on whether CBD was used in the diet or not and on the administration of LPS to the cockerels. The addition of CBD to the feed significantly reduced the level of total collagen in the livers by several times (CON and CH2). Furthermore, the serum levels of type III collagen also decreased only after CBD was administered. The reduction in the proportion of total collagen in the liver may indicate that CBD has a therapeutic effect on liver tissue, even providing protective effects against induced LPS stress. For pectoral muscle tissue, CBD administration alone was also not found to impair the quality of the pectoral muscle, which is consistent with previous reports by our team [Konieczka *et al.* 2022].

In another study from the same research, CBD administration alone had the effect of increasing the concentration of total collagen in the pectoral muscle, which contrasts with its effect in liver tissue. Additionally, a previous study conducted by our team reported that the addition of CBD to feed did not significantly affect the collagen content of intestinal tissue, and there was no significant effect observed on total collagenase enzyme concentrations in the intestines of 35-day-old birds following *C. perfringens* provocation [Konieczka *et al*. 2020]. These findings may indirectly explain the changes observed in tissue collagen concentration.

Consumer interest in products of high health-promoting quality has led to attention being paid to their enrichment with fatty acids, including those of the n-3 family; this has mainly concerned meat and eggs. The enrichment of animal products with n-3 fatty acids is achieved mainly through the use of linseed, flaxseed oil, or oils derived from marine and oceanic products, in animal diets [Moghadasian 2008, Palmquist 2009, Yeung *et al*., 2019]. Sources of fatty acids that are among the most important in terms of the human diet can be hemp seeds and their products, including hemp oil, which contains CBD and is found in chickens' diets, among others. Chicken meat and its most valuable component, the breast muscle, is considered one of the main sources of PUFAs in the human diet; and the fatty acid profile of such meat can be modulated by diet and the degree of fat absorption [Hulan *et al*. 1989, Poorghasemi *et al.* 2013]. In the present study (only the most significant interactions are presented), the addition of CBD to chicken feed alone significantly altered the ratio of n-6 to n-3 fatty acids. Only the LPS-induced stress and the administration of CBD to feed showed similar proportions of n-6/n-3 to the non-supplemented CBD group. Importantly, the application of LPS and CBD to feed has a significant effect on increasing n-3 acids in the breast muscle, which is positive from a dietary perspective, but may pose a risk to human health as LPS is bound to the cell wall of *E. coli*. The administration of CBD to chicken feed alone does not adversely affect the levels of PUFA, n-3 and n-6 in breast muscle. Hemp oil itself, which contains CBD, is rich in fat (25-35 g/100 g). Hemp oil contains significant amounts of linoleic acid (LA, 18:2, n-6), which accounts for more than half of the total FA. In addition, the composition includes approximately 16% α-linolenic acid (ALA, 18:3, n-3), about 12% oleic acid (OA, 18:1, n-9), approximately 6% palmitic acid (PA, 16:0), and around 3% γ-linolenic acid (GLA, 18:3, n-6), among several other fatty acids [Callaway 2004, House *et al*. 2010, Vonapartis *et al*. 2015, Kanbur 2022]. Analyzing the fatty acid profile of CBD-enriched hemp oil, it seems reasonable to change the fatty acid profile in the pectoral muscle precisely in the direction of increasing the concentration of n-6 fatty acids and thus the n-6/n-3 ratio.

It was found that feeding a diet of CBD to chickens infected with *Clostridium perfringens* reduced the levels in meat of volatile compounds that are correlated with bacterial activity [Konieczka *et al*. 2022] and that *C. perfringens* affects lipid metabolism by down-regulating the expression of the genes associated with fatty acid catabolism, including peroxisome proliferation-activated receptor alpha,

carnitine palmitoyltransferase 1, and acyl-CoA oxidase 1 [Zhou *et al.* 2016] which play important roles in lipid metabolism and thus strongly contribute to meat sensory attributes. Analyzing the key fatty acids that pertain to the human diet (i.e., LA, ALA, DHA, and PUFA), which were the focus of this study (only the most important interactions are presented), it was discovered that the inclusion of 3.0% CBD in feed markedly decreased the levels of C18:2, C20:4, C22:6, n-3, and n-6 acids. Interestingly, the presence of the C18:3 n-6 acid was detected under induced stress conditions. For the CON group, the addition of CBD to the feed had no impact on the levels of this acid in the liver tissue; while the amount of this fatty acid was found to be below the detection threshold (0.050 g/100 g). Delta6-desaturase facilitates the formation of GLA, which is the first product in the conversion of linoleic acid into dihomo-γ-linolenic acid (DGLA) and arachidonic acid (AA). DGLA serves as a direct precursor to prostaglandin series 1 (PGE1) as well as thromboxane and leukotriene series 3 (TBX3 and LT3, respectively) whereas AA serves as a direct a precursor to prostaglandin series 2 (PGE2), thromboxane, and leukotriene series 4 (TBX4 and LT4, respectively). Prostaglandin E1 binds to surface receptors in smooth muscle, which results in an increase in intracellular cAMP (cyclic adenosine monophosphate) levels. Conversion to PGE1 provides γ-linolenic acid with anti-inflammatory and antiproliferative effects, as well as a possible lipid-lowering capacity [Hornych et al. 2002]. Delta 6-desaturase, an enzyme found in humans and birds in small quantities [Lands *et al*. 1990], is most prominently expressed in the liver, brain (specifically in neurons and astrocytes), lung parenchymal cells, cardiomyocytes, and retinal cells of humans. The body's pathological states may also modify Δ6-desaturase activity. Infections may elucidate the presence of these fatty acids in the livers of cockerels when exposed to induced stress, in quantities exceeding the detection threshold of the fatty acid profile assay used [Fan and Chapkin 1998].

When broiler chickens are infected, various biochemical changes may occur in their bodies, including increased activity by enzymes like AST. AST is found in liver cells, muscles, hearts, and other tissues, and increased activity can arise from cellular damage or oxidative stress linked to the infection. Several studies have revealed that LPS can provoke an inflammatory response in broilers [Zhang *et al.* 2020]. Plasma AST and ALT activities are commonly used in clinical settings as specific markers of liver damage [Senior 2012]. Our findings suggest that administering CBD alone to the diet of cockerels does not significantly impact changes in serum AST levels. However, when LPS is orally administered to induce stress, and CBD is used in the diet, an increase in AST activity is observed. This is attributed to the body's response to the lipopolysaccharide of the bacterium *E. coli*. Keratin kinase (CK) is an enzyme present in muscle and other tissues that facilitates the conversion of creatine and phosphocreatine, which are essential for energy storage and transport in muscle cells. Elevated serum CK activity is indicative of muscle damage, with levels being influenced by a range of factors, such as injury, disease, or stress. Concerning broiler chicken rearing, the muscular health of chickens can be negatively affected by stress

related to rearing conditions, infections, or other stressors. Elevated CK activity may suggest muscle stress. Creatine kinase (CK) is a fundamental enzyme in cellular bioenergetics, and supports cellular and ATP homeostasis. Augmented CK levels are linked to speedy creatine phosphate (CP) clattering, glycolysis, accelerated pH diminution, and elevated water loss [Zelechowska *et al*. 2012]. Our research suggests that incorporating CBD into the diets of chickens leads to a decrease in serum CK levels and provides protection against *C. perfringens*, as evidenced by the reduced CK levels in CH1 cockerels. No muscle defects were detected upon dissection of the carcasses, nor were there any changes in the chemical composition of the breast muscle, which may suggest that the intensive production system and the fast-growing genetic material of Ross 308 lines influences CK activity and the potential therapeutic effects of CBD in reducing serum CK levels.

Analyzing the results we obtained regarding the improvement of the antioxidant capacity of CBD, we did not find that the addition of CBD to feed increased the activity enough to inhibit free radicals. Nevertheless, it is worth noting that in the case of the CH1 and CH2 groups, where the chickens' organisms were exposed to subclinical doses of *C. perfringens* and LPS, no significant differences were found either; which allows us to conclude that the addition of CBD to the feed under induced stress conditions, nevertheless, resulted in the maintenance of the oxidative potential at the CON group level. The mechanisms behind this may be attributed to the better radical suppression capacity of hemp oil containing PUFAs, 9 γ-tocopherols, or CBD [Vispute *et al*. 2021].

Conclusions

The use of a 3.0% *Cannabis sativa* extract supplement in chickens' diets increases the final body weight of chickens and maintains a high final body weight when birds are exposed to stress-induced conditions, while maintaining good results for slaughter analysis. The use of the investigated extract in the chickens' diet does not adversely affect the physicochemical parameters and basic chemical composition of the breast muscle and liver, maintaining their characteristic features. Although the use of CBD in chickens' diets may increase the n-6/n-3 acid ratio, this difference is not so large that, from the human diet's perspective, the addition of CBD negatively impacts human health. In addition, the use of a 3.0% *Cannabis sativa* extract supplement in chicken nutrition allows for the maintenance of blood biochemical and antioxidant parameters under stress-induced conditions, which contributes to maintaining the homeostasis of the chicken body. The 30 g/1000 g *Cannabis sativa* extract can provide a protective element to improve rearing under demanding conditions of intensive animal production.

Disclosures

The authors declare no conflict of interest.

Acknowledgments. *The manuscript is a part of the PhD thesis of Damian Bień.*

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