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Effects of dietary protein and *Macleaya cordata* alkaloid extract supplementation on growth performance, intestinal morphology, immunological responses, serum biochemical metabolites, and the ilealmicrobiota in broiler chickens

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ABSTRACT

Background & Aim: This study investigated the effects of feeding a phytogenic chemical, *Macleaya cordata* alkaloid extract (MCAE), on growth performance, intestinal morphology, immune responses, biochemical metabolites, and ilealmicrobiota in broiler chickens fed two levels of crude protein (CP).
Experimental: A total of 560 one-day-old male (Ross 308) broilers were randomly assigned in a 2×4 factorial arrangement, with8 dietary treatments and 5 replicates per treatment. Two concentrations of dietary CP (100 and 95 % of the recommended requirements) and four inclusion levels of MCAE (0, 180, 360, or 540 mg/kg diet) were applied for 5 weeks.
Results: At 35 d, the results showed that supplementation with 540 mg/kg of MCAE in both CP levels resulted in greater ($P \leq 0.05$) body weight than the control group. Also, increasing the dosage of MCAE improved broiler chickens' average daily weight gain and feed conversion ratio. The albumin to globulin ratio and relative weights of the spleen and bursa were not affected by dietary CP and MCAE supplementation. The results indicated that 360 mg/kg MCAE supplementation significantly ($P<0.05$) reduced the heterophil-to-lymphocyte ratio compared to the control group. Increasing levels of MCAE supplementation increased blood cholesterol and triglyceride levels linearly ($P<0.05$). The ileum villus height: crypt depth ratio in chickens fed low-protein diets was greater than the control diet ($P<0.01$). The coliform bacteria count of ileum on day 15 was not affected by dietary treatments but increasing the MCAE supplementation level from 0 to 540 mg/kg decreased the coliform count of broiler chickens numerically. When the levels of MCAE supplementation were increased on day 28, antibody titers against Newcastle disease increased numerically.
Recommended applications/industries: Finally, our findings indicated that supplementing broiler chicken diets with 540 mg/kg MCAE can improve performance and immunological responses.

1. Introduction

Antibiotic growth promoters (AGPs) have been used in pig, poultry, aquatics, and ruminant diets at sub-therapeutic doses to improve growth performance and protect health (Sneeringer *et al.,* 2020). However, the use of antibiotics as growth promoters in animal feed has been banned due to rising public concern over the rise of antibiotic resistance in zoonotic bacterial infections, which presents a hazard to public health

(Landy and Kheiri, 2023; Afiouni *et al.*, 2023; Landy *et al.*, 2018; Kavyani *et al.*, 2014). Many studies have been done to find antibiotic alternatives with comparable antibacterial and growth-promoting benefits without causing bacterial resistance. Phytochemicals have been recognized as possible antibiotic alternatives. Phytobiotics refer to a wide variety of plant-derived compounds such as necessary oils, herbs, and oleoresins which have been more frequently used in poultry feeds (Windisch *et al.*, 2008). Phytochemicals like the quaternary benzophenanthridine alkaloids (QBA) sanguinarine and chelerythrine, as well as protopine alkaloids (PA), pulled out from plants like *Macleaya cordata*, have properties of antimicrobial, anti-inflammatory, anti-fungal, adrenolytic, as well as sympatholytic properties so they canimprove growth performance of birds (Niu *et al.*, 2012).

These bioactive chemicals may also increase villus width, villi exterior space in the jejunum, and villus height: crypt depth ratio (VH/CD) in the ileum of broiler chickens (Reansoi *et al.*, 2015), and laying hens (Bavarsadi *et al.*, 2017). Phytogenic feed additives may stimulate the release of digestive enzymes, bile, and mucus, which may help improve gut function (Abdelli *et al.*, 2021). On the other hand, dietary supplementation with sanguinarine preparation decreased villus height (VH) in the duodenum of broiler chickens (Jankowski *et al.*, 2009). In addition, several studies have shown that supplementing broiler chickens' diets with isoquinoline alkaloids enhanced and controlled their immune systems and immunological activities. Additionally, sanguinarine has been demonstrated to enhance antibody titer against sheep red blood cells (SRBC) in broiler chickens (Mathivanan *et al.*, 2007; Lee *et al.*, 2015). Investigations have shown that this pharmaceutical molecule may activate phagocytosis, and cause the host to react defensively (Gudev *et al.*, 2004). Sanguinarine has been shown to inhibit the development of bacteria that generate gastrointestinal distress in broiler chickens (Yakhkeshi *et al.*, 2011; Pickler *et al.*, 2013), laying hens (Bavarsadi *et al.*, 2017) and piglets (Chen *et al.*, 2019). Parameters related to growth performance in broiler chicks are also influenced by the dietary concentration of crude protein (CP), which might affect carcass yield (Abbasi *et al.*, 2014). However, little is known about the effects of dietary *Macleaya cordata* alkaloid extract (MCAE) supplementation on intestinal morphology, immune responses, and ilealmicrobiota of broiler chickens fed with a low-crude protein diet. The present experiment aimed to show how dietary MCAE supplementation affects performance in broiler chickens fed different levels of CP. We anticipated that MCAE might increase growth performance in broiler chickens by improving intestinal morphology, immunological response, serum biochemical metabolite, and total bacterial count of the gut.

2. Materials and Methods

2.1. Birds and diet preparation

The animal care and use committee of the Isfahan Agricultural and Natural Resources Research and Education Center approved the experimental procedures (protocol No. 2594-264-1). The research was carried out at the Isfahan Agricultural and Natural Resources Research and Education Center's research farm in Isfahan, Iran. A total of 560 one-day-old male broiler chickens (Ross 308) were obtained from a commercial local hatchery and used in this experiment. Upon arrival, chicks were weighed and randomly assigned to 20 cages. Five replicate cages were randomly allocated to eight dietary treatments based on a factorial (2 × 4) completely randomized design. Experimental treatments included two levels of CP (CON; control diets at 100% of requirements and LCP; low-CP diets at 95% of requirements). The dietary treatments were supplemented with0, 180, 360, and 540 mg/kg MCAE (Sangrovit X10®, Animal Nutrition Center, Phytobiotics Futterzusatz stoffe GmbH, Eltville, Germany). During starter (1 to 15 days) and grower (16 to 35 days) periods, two diets were formulated to be iso-caloric and to meet or exceed nutrient requirements recommended by the Ross manual (Aviagen, 2019)(Table 1). *Macleaya cordata* alkaloid extract (MCAE) is acommercial product that includes a combination of QBA and PA and additional bioactive substances, including sanguinarine as well as chelerythrine alkaloids. Broiler chickens had free access to feed and water throughout the experiment. All experimental diets were fed in mash form. The birds were reared in a power-ventilated broiler house equipped with 40 battery cages (length 124 cm × width 65 cm) in an ecologically controlled broiler facility. During days 1 to 15 and 16 to 35, birds' densities ranged from 7.7 to 23.4 kg/m^2, respectively. The lighting schedule consisted of 23 hours of light and 1 hour of darkness. The lighting was provided by an incandescent lamp, with a light intensity of 30 lux. For days 1 to 3, the broiler house temperature was kept at 31 °C, then progressively decreased to 24 °C by the end of the fifth week. The relative humidity was kept at 50 to 70%.

Table 1. Ingredients and chemical composition of the basal diets during the starter (1 to 14 d) and grower (15 to 35 d) periods (as-fed basis).

Item	Dietary CP (% Requirement)			
	100% of the recommended CP		95% of the recommended CP	
	Starter	Grower	Starter	Grower
Ingredients (g/kg)				
Yellow corn, 8% CP	502	572	542	603
Soybean meal, 44% CP	415	345	380	318
Soybean oil	34.0	37.5	29.0	33.5
Limestone	16	16	16	16
Monocalcium phosphate	16.0	12.4	16.0	12.4
DL-Methionine	3.7	3.4	3.6	3.1
L-Lysine HCl	2.1	2.4	2.3	2.5
L-Threonine	1.2	1.2	1.1	1.1
Sodium chloride	3.5	2.3	3.5	1.6
$NaHCO_3$	-	1.8	-	2.8
Vitamin and mineral premix [1]	5	5	5	5
Choline chloride	1.5	1.0	1.5	1.0
Total	1000	1000	1000	1000
Calculated chemical composition (g/kg)				
Metabolizable energy (MJ/kg)	12.13	12.55	12.13	12.55
Crude protein	225	200	213	190
Calcium	10.0	9.1	10.0	9.1
Available P	5	4	5	4
Sodium	1.6	1.6	1.6	1.6
DEB [2] (meq/kg)	231	220	216	220
Apparent ileal digestible (g/kg)				
Lysine	12.8	11.4	12.2	10.8
Methionine	6.6	6.0	6.3	5.7
Methionine and Cystine	9.5	8.7	9.0	8.3
Threonine	8.1	7.3	7.7	6.9
Tryptophan	2.5	2.2	2.4	2.1
Arginine	13.9	12.8	13.2	12.2
Valine	9.5	8.8	9.0	8.4
Isoleucine	8.6	7.9	8.2	7.5

[1] Vitamin and mineral premix provided per kilogram of diet: vitamin A (retinyl acetate), 12,000 IU; cholecalciferol, 5,000 IU; vitamin E (alpha tocopherol acetate), 70 IU; vitamin K_3 (menadione), 3.5 mg; thiamin, 3.2 mg; riboflavin, 8 mg; pyridoxine, 4.3 mg; cyanocobalamin, 0.025 mg; niacin, 65 mg; D-pantothenic acid, 30 mg; choline chloride, 800 mg; folic acid, 2 mg; biotin, 0.3 mg; Fe, 70 mg as $FeSO_4.H_2O$; Cu, 16 mg as $CuSO_4.5H_2O$; Mn, 120 mg as MnO; Zn, 110 mg as $ZnSO_4.H_2O$; Se, 0.35 mg as Na_2SeO_3; I, 1.3 mg as KI. [2] Dietary electrolytebalance: (Na^+, meq/kg + K^+, meq/kg) – Cl^-, meq/kg.

2.2. Growth performance and carcass traits

The average body weight (BW) of broilers in each cagewas measured on days 15 and 35. To compute the FCR, the daily feed intake (DFI) and average daily weight gain (DWG) were obtained for each period (starter and grower). On day 35 of the experiment, two broiler chickens from each cage were randomly selectedand individually weighed and killed then the internal organs were separated, weighed and reported as a percentage of live body weight. Each chicken's small intestine was also removed, and the length was calculated from the gizzard to the ileocecal junction.

2.3. Hematological and biochemical parameters

The blood samples were obtained from the brachial veinon day 15 of age for hematological and biochemical parameters. The blood samples were taken from each chicken and immediately transferred to tubes containing EDTA as an anticoagulant for hematological profile assessments. May-Greenwald-Giemsa stain was used to make blood smears, and the heterophil to lymphocyte (H/L) ratios were calculated by counting 100 cells. Also, to evaluate the biochemical parameters the blood samples were centrifuged at 3000 rpm for 15 minutes at 4°C. Total cholesterol, total triglyceride, high-density lipoproten (HDL), and low-density lipoprotein (LDL) were measured in serum samples using auto analyzer equipment (BT3500, Biotecnica, Italy) (Gross and Siegel, 1993).Electrophoresis on cassettes of gelled cellulose-acetate (Cellogel®, MALTA Chemetron, Milan, Italy) was used to measure the concentration of protein fractions (albumins, alfa-1, alfa-2, beta-, and gamma-globulins) and determined by Global-scan densitometer (MALTA Chemetron, Milan, Italy). The clinilab analyzer was used to determine the total protein content using a colorimetric approach. The albumin to globulin (A/G) ratios was accordingly calculated (O'Connell *et al.*, 2005).

2.4. Immunity

The broilers were vaccinated against Newcastle Disease virus (NDV) and Infectious Bronchitis virus (IBV) via B1 H120 on the first day of the experiment. The commercially oil-adjuvant injectable emulsion against NDV and Avian Influenza virus (AI) was used (H9N2 subtype) for vaccinating broiler chicks, and they were injected subcutaneously with 0.2 mL per chick on day 9 of age. Also, chicks were orally vaccinated against NDV (Lasota) on day 21 of age. On days15 and 28 of age, serum sampleswere collected from 2 chicks from each cage, and were used to analysis of antibody titersagainst NDV and AI, via the hemagglutination inhibition test (HI), and HI antibodies were converted to $\log_2$. On day 22 of the experiment, 2 chicksper penwereinoculated via the brachial vein with 1 ml of 1% sheep red blood cells (SRBC) suspension. On day 28 of age, blood samples were taken and plasma was collected. Antibody titers against SRBC were measured by the microtiter procedure described by Wegmann and Smithies (1966). Titers were expressed as the $\log_2$ of the reciprocal of the highest dilution giving visible hemagglutination.

2.5. Morphology of small intestine

On day 35of age, two birds per cage were killed by cervical dislocation, and their digestive tracts were removed. Two tissue samples from the middle areas of the duodenum and jejunum were taken from each bird for histological investigations. A three-centimeter section of the duodenum (from the center of the duodenal loop to the pancreatic and bile ducts) and jejunum (from the bile duct entrance to Meckel's diverticulum) were obtained. Intestinal part specimens were removed and washed with phosphate buffer saline (PBS; pH 7.4) to remove all the contents. For 24 hours, gut parts were fixed in 10% neutral buffered formalin solutions. Paraffin wax was used to embed tissues regularly. A Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany) was used to cut longitudinal slices with a thickness of 5 µm which then placed them on glass slides. Thereafter, according to the method described by Uni *et al.* (1995), all samples were progressively dehydrated and stained with hematoxylin and eosin. The morphological evaluation of small intestine samples was performed using an optic microscope (Olympus CX31, Tokyo, Japan). The following parameters were calculated: villus height (VH), villus width (VW), and crypt depth (CD). The VH to CDratio was calculated accordingly. The villus surface area (VSA) was calculated as: $(2\pi)(VW/2)(VH)$ (Santos *et al.*, 2015).

2.6. Microbiota population of ileum

On day 15, two birds per cage were killed by cervical dislocation, and the digestive system was removed. Ilealdigesta specimens (1 gram) were collected individually in sterile containers from Meckel's diverticulum to 1 cm above the ileo-cecal conjunction. The samples were promptly transported to the microbiology laboratory on ice packs. For bacterial enumeration, 1 g sample of fresh ileal digesta was transferred into 9 mL PBS and serially diluted 10-fold in PBS. Diluents were next plated on eosin methylene blue agar (EMB, Merck, Germany) at 37°C for 24 hours to count *Escherichia coli (E. coli)* colonies, and the metallic green sheen settlements were counted to calculate the count of *E. coli* (CFU/g) colonizing the ileal digesta. Ileal digesta from chickens were plated onto xylose lysine deoxycholate agar (XLD, Oxoid, Basingstoke, UK) and cultured for 24 h at 37 °C to isolate *Salmonella*. The colonial counts (*E. coli* and *Salmonella*) were calculated as log10 CFU/g of ileal digesta (Islam *et al.*, 2014).

2.7. Statistical analysis

The data were analyzed for standard distribution as a 2 × 4 factorial arrangement in a completely randomized design utilizing a 2-way general linear modelprocedure of SAS (2010) to determine the main effects (CP and MCAE)and the interaction effects (CP× MCAE) on measured parameters.The means were compared by Tukey's test ($P<0.05$).

3. Results and discussion

3.1. Growth performance, carcass characteristics, and intestinal traits

The main and interaction effects of MCAE and CP on the performance of broiler chicken have been shown in Table 2. On day 35 of age, the LCP group showed a significant increase in DFI compared to the CON group (P<0.04). The differences in BW in broilers fed diets containing 540 mg MCAE/kgon day 35 were higher than the control group (P<0.05). However, on days15 and 35of age no interaction was observed between CP and MCAE for BW, DFI, DWG, and FCR parameters (P>0.05).

Table 2. Effects of dietary crude protein level and *Macleaya cordata* alkaloid extract supplementation on growth performance of broiler chicken during different growth periods.

Treatment		Performance parameters				
		Body weight (g), 15 d	**Body weight (g), 35 d**	**Average daily weight gain (g)**	**Average daily feed intake (g)**	**FCR[1]**
Main effects						
CP[1](%)						
100		487	2256	63	93.9[b]	1.47
95		493	2259	63	94.2[a]	1.49
MCAE [1](mg/kg)						
0		477	2223[b]	62	93.0	1.49
180		495	2253[ab]	63	93.5	1.48
360		492	2259[ab]	63	94.3	1.49
540		497	2296[a]	64	94.2	1.47
Interactions						
CP (%) × MCAE (mg/kg)						
100	0	472	2220	61	93.1	1.50
100	180	492	2252	63	92.3	1.46
100	360	487	2261	63	94.2	1.49
100	540	498	2291	64	93.6	1.45
95	0	482	2226	62	92.9	1.49
95	180	498	2253	63	94.8	1.50
95	360	497	2258	63	94.5	1.49
95	540	494	2300	63	94.8	1.49
		P-Value				
CP		0.47	0.86	0.94	0.04	0.14
MCAE		0.28	0.05	0.16	0.13	0.44
CP×MCAE		0.90	0.99	0.95	0.14	0.29
Pooled SEM [2]		10.9	25.55	0.78	0.63	0.01

[1]FCR: feed conversion ratio, CP: crude protein, MCAE: *Macleaya cordata* alkaloid extract.
[2]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different (P≤0.05).

Table 3 shows the impacts of dietary CP, and the CP×MCAE interaction analysis on carcass components and intestine characteristics. The relative weights of the spleen and bursa were not altered by dietary CP and MCAE supplementation (P>0.05). Relative weights of ceca, small intestine, and smallintestine length, and theinteraction effects (CP and MCAE supplementation) were not significantly different (P>0.05). Dietary MCAE supplementation decreased the ceca weight (P<0.01), but CP concentration and MCAE supplementation did not change the intestinal weight or length (P>0.05).

Based on our results, MCAE supplementation improved performance criteria in the present experiment. Our findings are consistent with the results obtained on broiler chickens and different kinds of animals which received different levels of MCAE in the diet (Zdunczyk *et al.*, 2010; Karimi *et al.*, 2014; Goodarzi Boroojeni *et al.*, 2018; Kikusato *et al.*, 2021; Wang *et al.*, 2022; Song *et al.*, 2023; Khongthong *et al.*, 2023). Nevertheless, the obtained results in the current study are in contrast with the results obtained by Vieira *et al.* (2008a), who found that broiler chicks fed diets containing 25 and 50 ppm of MCAE had better FCR. The ingredients of the experimental diets, the age and sex of the birds, the dose of MCAE, and the utilized alkaloid extraction process, may vary QBA and PA concentration. All the precise mechanisms underlying the role of phytobiotics as animal growth promoters have not yet been completely elucidated. One of the possible mechanisms by which MCAE could increase chicken growth performance is upregulating neurotransmitters. For example, research found that MCAE may enhance DFI in broiler chickens by upregulating serotonin production and secretion (Tschirner *et al.*, 2003). As a result, an increase in DFI found with MCAE supplementation in the current research may increase serotonin synthesis in the birds. These lines of evidence suggest that MCAE supplementation may restore intestinal barrier function by reducing corticosterone secretion, thereby improving DFI in broiler chickens (Kikusato *et al.*, 2021). On the other hand, the better growth performance that has been observed in the present research may be partly related to MCAE's anti-inflammatory capabilities since MCAE has been shown to suppress the activation of NF-κB, a vital inflammatory controller. Sanguinarine may have direct and indirect anabolic activity on target tissues via the activation of endocrine and antioxidative defense systems. In the current research, supplementation of MCAE increased DFI and growth performance, as assessed by contrast analysis. Valenzuela-Grijalva *et al.* (2017) reported that the positive effects of MCAE have been linked to increased appetite, enhanced nutritional digestibility, and presumably lower inflammation. If phytobiotics and their metabolites mitigate inflammatory status and subsequent protein degradation, amino acid utilization for such protein synthesis would no longer be necessary and normal muscle growth may progress. This could be one of the mechanisms that promote the growth performance of broilers (Kikusato *et al.*, 2021).

The decreased ceca relative weight which was observed with MCAE supplementation may also be associated with a general improvement in intestinal and cecal health, as MCAE supplementation can improve the cecal environment by modulating the cecal microbiota population in favor of butyric acid-producing microbes. The presence of high butyric acid in the chicken ceca is associated with reduced colonization of *Salmonella* pathogenic species (Zdunczyk *et al.*, 2010). In addition, enhanced digestibility of CP in the upper intestine may have contributed to the lower ceca relative weight in the current research. Thus, in the present investigation, MCAE supplementation may have repressed cecal fermentation, resulting in decreased relative cecal weight owing to reduced cellular hypertrophy and hyperplasia (Mateos *et al.*, 2012). The digestive capability of the gut is linked to the relative length of the intestine since a longer intestine may represent a larger exterior space for absorption (Wijtten *et al.*, 2012). Feeding MCAE raises the relative length of the small intestine while decreasing its relative weight, according to Lee *et al.* (2015). On the other hand, some studies showed that low dietary CP in diets decreased jejunum and ileum length. The decrease in jejunum and ileum length observed in low-CP diets might be attributed to the reduced absorption of nutrients relative to the control group, which may explain the lower body weight of the birds (Ajao *et al.*, 2022). However, feeding MCAE or CP level had no effect on the present investigations of intestinal relative length and weight. The impacts of MCAE supplementation on the structure of the intestinal mucosa and its association with nutrient digestibility and development implementation in broiler chickens require further investigation. MCAE treatments decreased the comparative weight of the ceca while not affecting the comparative weight of the intestine. The relative weight of the spleen and bursa of fabricius were not influenced by feeding dietary supplementation of MCAE or CP level (Table 3).

The present result was supported by the previous studies that dietary sanguinarine had no significant effect on the weight of the lymphoid organ of the broiler (Lee *et al.* 2015). On the other hand, Wang *et al.* (2022) reported that supplementation of sanguinarine in the diet of broiler chickens can significantly increase the relative weight of the spleen during heat stress. This shows that phytobiotics may be effective in the diet on stressful situations in broiler chickens.

Table 3. Effects of dietary crude protein level and *Macleaya cordata* alkaloid extract supplementation on carcass and intestine characteristics of broiler chicken during different growth periods.

Treatment		Carcass components [1]		Intestine components[1]		
		Spleen	**Bursa**	**Ceca**	**Intestine**	**Intestine length [2]**
Main effects						
CP[3](%)						
100		0.10	0.06	0.69	3.90	8.08
95		0.09	0.06	0.66	3.98	8.19
MCAE [3](mg/kg)						
0		0.10	0.06	0.77[a]	3.90	8.15
180		0.09	0.07	0.70[ab]	3.84	8.17
360		0.01	0.06	0.60[b]	3.97	8.29
540		0.09	0.06	0.63[b]	4.04	8.18
Interactions						
CP (%)× MCAE (mg/kg)						
100	0	0.11	0.06	0.80	3.73	8.10
100	180	0.09	0.08	0.70	4.04	8.30
100	360	0.10	0.07	0.60	3.81	8.30
100	540	0.10	0.06	0.60	4.03	8.20
95	0	0.09	0.07	0.80	4.07	8.30
95	180	0.09	0.08	0.70	3.64	8.00
95	360	0.10	0.06	0.60	4.14	8.30
95	540	0.10	0.06	0.60	4.07	8.27
		P-Value				
CP		0.32	0.14	0.51	0.49	0.92
MCAE		0.33	0.35	0.01	0.58	0.41
CP×MCAE		0.28	0.07	0.81	0.08	0.71
Pooled SEM [4]		0.004	0.006	0.05	0.06	4.49

[1] Carcass components and intestine components (ceca and intestine) were weighed and expressed as a percentage of live body weight.
[2] Values are expressed as relative length (cm/100 g BW).
[3]CP: crude protein, MCAE: *Macleay acordata* alkaloid extract.
[4]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different (P≤0.05).

3.2. Immune responses

Table 4 indicates the impacts of dietary CP and the CP×MCAE interaction analysis on antibody titers against NDV,AI, and SRBC. Dietary CP consumption did not affect theantibody titers (P>0.05). On days15 and 28 of age, the main and interaction effects of CP and MCAE were not significant for antibody titers against NDV, AI, and SRBC (P>0.05). However, antibody titers against NDV increased numerically when the levels of MCAE supplementation were increased on day 28.

A comparison of broilers fed MCAE-free diets with broilers fed diets containing MCAE showed that the antibody titers against NDV increased. Our results indicated that decreasing CP content in the diet of broiler chickens had no significant effect on serum antibody titers against NDV and SRBC. In agreement with our findings, Abbasi *et al.* (2014) found that a reduction in dietary CP content in broiler chickens had no suppressive effects on serum antibody titers against NDV and SRBC in broiler chickens. Our results are in agreement with the results of Karimi *et al.* (2014), who found that feeding broiler chickens with diets containing sanguinarine increased antibody titers against NDV. In addition, antibody titers against SRBC in broiler chicks that fed diets containing 180 mg MCAE/kg, increased quantitatively compared to the control group. Our findings are consistent with the findings of Mirzadeh *et al.* (2012) and Kosina *et al.* (2004), which did not observe any significant effect of sanguinarine on antibody titers against SRBC in birds and pigs. In contrast with our finding, Bavarsadi *et al.* (2017) reported that the antibody titer against SRBC in laying hens fed 3.75 mg MCAE/kg of diet was significantly increased compared to the control group. Yakhkeshi *et al.* (2011) also found that serum antibody titers against SRBC were improved in broiler chickens. Several studies have shown that the addition of *Macleaya cordata* extracts to the feed can improve serum immune indices in broiler chickens, pigs, and laying hens (Song *et al.*, 2023; Liu *et al.*, 2022; Zhang *et al.*, 2022; Liu *et al.*, 2016; Yakhkeshi *et al.*, 2011).

Table 4. Effects of dietary crude protein level and *Macleaya cordata* alkaloid extract supplementation on immune responses of broiler chickens at different growth periods.

Treatment		Antibody titers (Log_2) Newcastle disease virus, 15 d	Newcastle disease virus, 28 d	Avian influenza virus, 28 d	SRBC[1], 28 d
Main effects					
CP[2](%)					
100		4.18	7.63	5.63	6.20
95		4.35	7.73	5.80	6.50
MCAE [2](mg/kg)					
0		4.30	7.50	5.80	6.30
180		4.35	7.80	5.70	6.65
360		4.25	7.70	5.65	6.25
540		4.15	7.70	5.70	6.20
Interactions					
CP (%) × MCAE (mg/kg)					
100	0	4.50	7.70	5.80	6.10
100	180	4.00	7.80	5.50	6.50
100	360	4.10	7.50	5.50	6.20
100	540	4.10	7.50	5.70	6.00
95	0	4.10	7.30	5.80	6.50
95	180	4.70	7.80	5.90	6.80
95	360	4.40	7.90	5.80	6.30
95	540	4.20	7.90	5.70	6.40
		P-Value			
CP		0.35	0.67	0.32	0.18
MCAE		0.88	0.84	0.94	0.48
CP×MCAE		0.22	0.58	0.78	0.95
Pooled SEM [3]		0.17	0.22	0.17	0.22

[1] SRBC = Sheep red blood cell.
[2]CP: crude protein, MCAE: *Macleaya cordata* alkaloid extract.
[3]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different ($P \leq 0.05$).

Besides, Guo *et al.* (2021) showed that dietary MCAE did not affect the serum levels of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) in laying hens. Immunoglobulin content is an indicator of the immune response of animals. Improved growth performance might be attributed to a more balanced immune system function. Furthermore, MCAE increased the levels of immunoglobulins, thus enhancing the immune function of broilers, indirectly inhibiting pathogenic bacteria in the intestine, reducing pathogenic bacterial metabolite production and damage to the intestinal epithelial cells, and ultimately protecting the intestinal mucosa. In addition, MCAE may exert a direct bacteriostatic effect (Newton *et al.*, 2002) and immuno-modulatory effects (Khadem *et al.*, 2014) but, more researches are needed to directly assess the level of immune system activity by dietary MCAE in broiler chickens.

3.3. Hematological, biochemical metabolites assessments

Table 5 shows the effects of feeding dietary MCAE and two levels of CP on serum indices and biochemical metabolites of broiler chicks on day 15 of age. On day 15, a significant interaction effect between CP and MCAE for blood cholesterol, triglyceride,and the H/L ratio was observed ($P<0.05$). MCAE supplementation increased blood cholesterol and triglyceride levels linearly ($P<0.05$). Nevertheless, interaction analysis demonstrated that dietary supplementation with MCAE and CP enhanced blood cholesterol compared to the control treatment ($P=0.05$). Additionally, dietary CP level did not affect the biochemical serum profile($P>0.05$). On the other hand, feeding low-CP diets resulted in numerically elevated serum triglyceride content ($P=0.12$). The A/G ratio was notaffectedby the experimental treatments ($P>0.05$).

The highest H/L ratio was observed in the 180 mg MCAE/kg group and the lowest H/L ratio was observed in the 360 mg MCAE/kg group (P<0.05). A significant interaction effect between CP and MCAE was observed for the H/L ratio on day 15 (P=0.01).

Table 5. Effects of dietary crude protein level and *Macleaya cordata* alkaloid extract supplementation on blood biochemical indices and hematological parameters of broiler chickens on day 15 of age.

Treatment		Blood biochemical factors and hematological parameters					
		Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	A/G ratio [1]	H/L ratio [2]
Main effects							
CP[3](%)							
100		187.5	129.0	105.0	58.0	0.90	0.42
95		187.7	187.7	102.6	59.8	0.95	0.41
MCAE [3](mg/kg)							
0		171.1 ^{b}	125.2 ^{b}	96.7	54.3	0.92	0.42 ^{a}
180		186.5 ^{ab}	114.1 ^{b}	105.0	60.5	0.94	0.44 ^{a}
360		191.8 ^{a}	143.2 ^{ab}	106.1	62.1	0.89	0.38 ^{b}
540		200.9 ^{a}	166.7 ^{a}	107.3	58.8	0.97	0.41^{ab}
Interactions							
CP (%)× MCAE (mg/kg)							
100	0	174.1 ^{b}	96.8 ^{c}	99.1	54.5	0.94	0.42 ^{b}
100	180	171.4 ^{c}	125.4 ^{bc}	90.9	55.7	0.86	0.49 ^{a}
100	360	203.9 ^{a}	155.7 ^{ab}	116.8	64.1	0.84	0.33 ^{c}
100	540	200.6 ^{ab}	138.4 ^{bc}	113.4	58.0	0.96	0.42 ^{b}
95	0	168.2 ^{c}	153.6 ^{ab}	94.4	54.2	0.90	0.42 ^{b}
95	180	201.7 ^{ab}	102.8 ^{c}	119.2	65.3	1.02	0.38 ^{bc}
95	360	179.8 ^{abc}	130.7 ^{bc}	95.5	60.2	0.93	0.42 ^{b}
95	540	201.2 ^{ab}	195.1 ^{a}	101.3	59.7	0.97	0.40 ^{b}
		P-Value					
CP		0.97	0.12	0.59	0.62	0.31	0.49
MCAE		0.03	0.01	0.35	0.46	0.74	0.03
CP×MCAE		0.05	0.01	0.01	0.60	0.54	0.01
Pooled SEM [4]		6.79	10.23	4.51	3.60	0.05	0.01

[1]A/G ratio: albumin to globulin ratio.
[2] H/L ratio:heterophil to lymphocyte ratio.
[3]CP: crude protein, MCAE: *Macleaya cordata* alkaloid extract.
[4]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different (P≤0.05).

The serum biochemical parameters are an indicator of the physiological function of the animal body, indirectly reflecting nutrient metabolism, and changes in organ functions and nutrient deposition (Wang *et al.*, 2009). Serum concentrations of HDL, LDL, and total cholesterol are important indicators of cholesterol metabolism. In the present study, MCAE supplementation significantly increased cholesterol and triglyceride levels and numerically increased HDL and LDL cholesterol. These findings are in accordance with the results of experiments which have been done by Yakhkeshi *et al.* (2011) and Liu *et al.* (2022), which reported that serum HDL, LDL, and total cholesterol levels were higher than the control group when broiler chicks fed with a diet containing *Macleaya cordata* extract, which helped to promote the growth performance. In our study, the results indicate that the addition of MCAE may promote the digestion and absorption of lipids, but it has a minimal effect on protein and carbohydrate metabolism in the diet. The enhanced deconjugation of bile acids results in higher bile acid excretion from the intestinal tract. Increased bile acid excretion encourages bile acid replenishment from cholesterol, so decreasing plasma cholesterol levels (Yin *et al.*, 2021). In addition, the herbal extract reduced acetyl Co-A carboxylase in the liver and tissues. Reduced lipogenesis in the liver may result in lower blood lipid levels. Herbal extracts in the diet have been shown to raise T3 hormone levels. In birds, this hormone stimulates metabolism, which may reduce serum lipids (Denbow, 2000). The mechanism of the effect of MCAE on lipid and protein metabolism needs further studies.

Despite the present findings, several studies demonstrated that dietary supplementation of sanguinarine could reduce serum cholesterol concentration but, serum HDL and triglyceride content were not affected in laying hens (Bavarsadi *et al.*, 2017). Furthermore, the addition of sanguinarine to the diet led to a reduction of serum triglyceride content but, total cholesterol was not affected (Aljumaah *et al.*, 2020). On the other hand, Manaa *et al.* (2022) found that the serum total cholesterol and LDL content in turkey were unaffected by Sangrovit supplementation but, serum triglyceride content was reduced by the administration of higher levels of Sangrovit in the diet. In addition, this study found that the serum HDL content was reduced by Sangrovit supplementation in turkey. However, conflicting results by Sangrovit on cholesterol levels have been reported in broiler chickens. It was speculated that essential oil components may have hypocholesterolemic effects in broiler chickens (Lee *et al.*, 2015). In the current study, we did not observe essential oil components mediated hypocholesterolemic effects. However, some reports showed that this parameter was not affected in Xuefeng black-bone chicken or broiler chickens fed with Sangrovit (Mirzadeh *et al.*, 2012; Hussein *et al.*, 2020; Liu *et al.*, 2020; Guo *et al.*, 2021; Zhang *et al.*, 2022; Wang *et al.*, 2022). Variations between these studies may be attributed to the differences in the composition of the used diet, age, and sex of the birds, dose of *Macleaya cordata* extract, plant extracts, and other bioactive constituents.

Our results indicated that the A/G ratio was unaffected by feeding low CP diets or dietary inclusion of sanguinerine. In agreement with our findings, Ferreira *et al.* (2023) and Kosina *et al.* (2010) found that the inclusion of sanguinarine had no influence on the A/G ratio in horses and pigs, respectively. However, Bavarsadi *et al.* (2017) reported that sanguinarine reduced the A/G ratio in laying hens. On the other hand, Yang *et al.* (1999) found that dietary *Macleaya cordata* extract increased A/G ratio in rats. Dietary inclusion of sanguinarine (360 mg/kg of diet) caused a reduction in heterophil percentage, a rise in lymphocyte percentage, and in turn a decline in H/L ratio compared to the control group. Our results are in agreement with the obtained results by Bavarsadi *et al.* (2017) who showed a decreased the H/L ratio in laying hens fed diets supplemented with sanguinarine. In contrast with our research, Yakhkeshi *et al.* (2011) found that dietary alkaloids elevated the H/L ratio. Heterophile to lymphocyte ratio serves as a suitable index of chronic and physiological tension and reflects the immune system status (Teirlynck *et al.*, 2009), and the suppression in this ratio as the consequence of increasing sanguinarine content might be associated with the inhibition of oxidative burst via preventing the formation of NADPH oxidase protein complex. Generally, the chickens with low H/L had enhanced intestinal immunity (Vrba *et al.*, 2004). In this study, using different levels of dietary CP had no remarkable effects on the H/L ratio.

3.4. Intestinal morphology and microbiota bacterial count

Table 6 shows the morphometric investigation of the jejunum and ileumon day 35 in broiler chickens. On day 35, there was no significant interaction between CP and MCAE on jejunum morphology (P>0.05). Significant changes were observed in VH in the jejunum of broilers fed LCP diets compared to the control group (P<0.05).On day 35, the main effects and the interaction effects had non-significant effects on the jejunum's CD and VH/CD ratio (P>0.05).

Dietary MCAE supplementation resulted in higher CD in the ileum (P<0.05); but did not significantly influence on VH of the ileumof broilers (P>0.05). The VH/CDratio in the ileum was increased by a reduction ofthe dietary CP consumption (P<0.05). There was significant interaction between CP and MCAEon VSA of the ileum (P<0.05). The broiler microbiota of ileum contents during the first 15 days of the experiment are shown in Table 7. No interaction or main effects were identified on the microbiota (*E. coli* and *salmonella*) count of ileum bacteria on day 15 (P>0.05).

Table 6. Effects of dietary crude protein level and *Macleaya cordata* alkaloid extract supplementation on villus height (µm), crypt depth (µm), villus height to crypt depth ratio and villus surface area (mm) in the small intestine in broiler chickens at different growth periods.

Treatment		Jejunum[1]				Ileum[1]			
		VH	CD	VSA	VH/CD	VH	CD	VSA	VH/CD
Main effects									
CP[2](%)									
100		695.8 [b]	123.9	249.0	5.98	409.5	112.8	131.8	3.71 [b]
95		785.4 [a]	132.8	266.9	6.29	454.5	108.2	145.0	4.33 [a]
MCAE [2](mg/kg)									
0		801.4	125.5	243.2	6.25	425.3	101.5[b]	145.6	4.19
180		712.4	133.1	259.1	5.76	438.3	121.5[a]	122.5	3.72
360		749.6	134.7	267.1	5.91	427.8	116.4[ab]	133.2	3.89
540		699.1	120.2	262.4	6.62	436.6	102.8[b]	152.1	4.29
Interactions									
CP (%) × MCAE (mg/kg)									
100	0	775.6	125.6	227.4	6.00	448.5	108.4	169.1[ab]	4.14
100	180	659.6	124.2	251.0	5.68	378.1	124.5	100.6[c]	3.10
100	360	691.2	122.2	276.0	6.01	421.6	121.2	128.6[bc]	3.54
100	540	656.8	123.6	241.6	6.21	390.0	96.90	128.7[bc]	4.05
95	0	827.2	125.4	259.0	6.50	402.1	94.60	122.1[c]	4.25
95	180	765.2	142.0	267.2	5.83	498.5	118.2	144.4[abc]	4.33
95	360	808.0	147.2	258.2	5.81	434.1	111.6	137.9[abc]	4.24
95	540	741.4	116.8	283.2	7.02	483.3	108.7	175.4[a]	4.52
		P-Value							
CP		0.01	0.27	0.33	0.26	0.11	0.40	0.24	0.01
MCAE		0.06	0.54	0.80	0.15	0.98	0.02	0.26	0.29
CP×MCAE		0.85	0.46	0.68	0.61	0.13	0.35	0.02	0.39
Pooled SEM [3]		28.1	7.94	18.1	0.28	27.2	5.33	11.2	0.23

[1] VH: villus height, CD: crypt depth, VH/CD: villus height to crypt depth ratio, VSA: villus surface area = (2π) × (VW/2) × (VH).
[2]CP: crude protein, MCAE: *Macleaya cordata* alkaloid extract.
[3]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different (P≤0.05).

Table 7. Effects of dietary crude proteinlevel and *Macleaya cordata* alkaloid extract supplementation on microbiota of ileum contents in broiler chickens at different growth periods.

Treatment		Microbiota count of bacteria [1], (Log CFU/g)
Main effects		
CP[2](%)		
100		2.55
95		2.60
MCAE [2](mg/kg)		
0		3.08
180		2.63
360		2.46
540		2.14
Interactions		
CP (%) × MCAE (mg/kg)		
100	0	3.00
100	180	2.64
100	360	2.34
100	540	2.21
95	0	3.15
95	180	2.62
95	360	2.57
95	540	2.07
	P-Value	
CP		0.92
MCAE		0.67
CP×MCAE		0.99
Pooled SEM [3]		0.54

[1] Such as *E. coli* and *Salmonella*in the ileum contents.
[2]CP: crude protein, MCAE: *Macleaya cordata* alkaloid extract.
[3]Pooled SEM: pooled standard error of the mean (n = 5 replicates/treatment).
[a-d] Values in the same row with different superscripts are significantly different (P≤0.05).

The development of small intestinal morphology is directly related to the absorptive capacity of animals. The VH, CD, and VH/CD ratio are important indices to evaluate the morphology of the small intestine and measure the capacity of the digestive tract. A greater VH increases the surface area of the small intestine and enzyme secretion. The higher ratio of the VH/CD is an indicator of the ability of the small intestinal tract to digest feed and absorb nutrients (Chen *et al.*, 2019). Previous studies have shown that the VH, VH/CD ratio or surface area of the jejunum was greater in broilers supplemented with *Macleaya cordata* compared with the control group (Zhang *et al.*, 2022; Reansoi *et al.*, 2015). However, broilers fed 0.7 mg/kg sanguinarine in diet had increased VH in the duodenum and jejunum, reduced CD in the jejunum and ileum, and increased VH/CD ratio in the ileum compared to other groups. The capacity of sanguinarine to increase the capacity of absorption of the small intestine and increase digestion and absorption was established by the shift in villi and crypt deepness (Liu *et al.*, 2020). Although, Wang *et al.* (2021) showed that feeding piglets with diets containing 50 mg/kg *Macleaya cordata* extract with 1000 mg/kg benzoic acid increased VH and the VH/CD ratio in the duodenum and VH in the ileum, but reduced the CD in the jejunum. However, no improvement in VH, CD, or surface area by dietary supplementation with sanguinarine has been reported in several experiments (Karimi *et al.*, 2014; Goodarzi Boroojeni *et al.*, 2018; Hussein *et al.*, 2020; Aljumaah *et al.*, 2020) which are in agreement with our data. Moreover, Bavarsadi *et al.* (2017) reported that the supplementation of 3.75 or 7.5 mg MCAE/kg increased the VH/CD ratio and decreased CD in the jejunum. It has been reported that broiler chickens fed diets supplemented with sanguinarine had increased relative jejunal and ileal lengths (Lee *et al.*, 2015). Interestingly, in our study, MCAE supplementation had limited effects on improving the intestinal morphology of broilers. This may be due to higher doses that have been used than ours, and the previous studies have mainly used additives in the feed. On the other hand, our results indicated that 180 mg/kg of MCAE in the diet of broiler chickens increased CD compared to the control group.

The gut microbiota is the largest symbiotic ecosystem in hosts and has been shown to play an important role in maintaining intestinal homeostasis. The microflora in the intestinal can be affected by pathogens, inflammatory cytokines, diet, exercise, and gastrointestinal peptides. In agreement with earlier findings, 540 mg/kg of sanguinarine supplementation reduced non-significantly the total ileum bacterial count. The *Macleaya cordata* extract was discovered to inhibit or kill *E. coli* and modulate essential activities, including peristalsis and intestinal pH in laying hens (Bavarsadi *et al.*, 2017). On the other hand, one research postulated that the non-absorbed fraction of phytobiotics may promote intestinal function or act as a prebiotic. Phytobiotics can affect the population of gut microflora by interfering with their metabolic activities (Martel *et al.*, 2020). Lee *et al.* (2015) reported that the addition of 20 ppm of sanguinarine to the diet of broiler chickens increased cecal lactic acid bacteria and so improved gut microbiota. Moreover, dietary supplementation of *Macleaya cordata* extract has been reported to primarily alter the microbiota of the front half of the intestine of chickens, promoting the proliferation of *Lactobacillus* and the concentrations of acetate, propionate, butyrate, and total SCFAs in the ileum and cecum and inhibiting the colonization of *Escherichia coli* in broiler chickens and piglet. At the same time, *Macleaya cordata* extract can change the metabolic pathways of amino acids, vitamins, and lipids, and increase the contents of butyrate and secondary cholic acid, thereby improving the growth performance and health status of broiler chickens (Huang *et al.*, 2018; Chen *et al.*, 2019; Guo *et al.*, 2021).

4. Conclusion

The present study showed that supplementing broiler dietswith 540 mg/kg of MCAE increased growth performance when dietary protein was provided at 95 % of the recommended level. The present data indicated that MCAE can improve broiler growth performance by enhancing jejunum morphology, but MCAE does not influence the total count of bacteriain the ileum. Also, in broiler chickens, increasing MCAE supplementation promoted antibody titers against NDVon day 28. The current data also suggested that providing higher levels of MCAE to broiler chickens diet improves the gut microbiota and tissue, and their general health.

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