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Archiva Zootechnica 25:2, 39-62, 2022
DOI: *10.2478/azibna-2022-0013*

Effects of dietary supplementation with herbal extract as methionine replacer on growth performance, meat composition, oxidative stability and liver gene expression in broiler chickens

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ABSTRACT

In the present study, an herbal feed additive was tested for partial-to-complete replacement of synthetic methionine in poultry diets, along with its effects on performance, breast and thigh meat chemical composition, oxidative stability during refrigerated storage and the expression of five target genes in liver. In a 35 days trial, 600 one-day-old male chicks were randomly allocated to 4 groups with 10 replicates. Birds in the control group were fed a regular maize–soybean-based diet that covered DL-methionine needs while the second group (Meth40) was similar to control but contained only DL – Methionine at 40% of control diet. Diet in third group contained DL-Methionine at 40% of control and the herbal feed additive Methiorep ™ (Meth40+Mrep) with extracts of *Boerhavia diffusa, Azadirachta indica, Vigna mungo* and *Trigonella foenum-graecum*. Diet of fourth group was formulated to totally replace DL Methionine by Methiorep ™ (Mrep). Body weight gain and feed consumption were weekly recorded. At the end of the trial, all birds were slaughtered and 2 chickens per pen were selected for meat and liver

sampling. The liver was tested for the expression of five target genes, namely Methionine synthase (MTR), Tyrosine aminotransferase (TAT), Spermidine synthase (SMS), Methionine sulfoxide reductase (MSRB1) and Betaine homocysteine S-methyltransferase (BHMT). The results showed that the Meth40 group had reduced body weight compared to the Meth40+Mrep group while the Control and Mrep groups had comparable weights. Feed intake and feed conversion ratio did not differ among the experimental groups. Carcass, breast and thigh meat yield were higher in the Mrep and the Control compared to Meth40 and Meth40+Mrep groups. Also, meat oxidation was significantly lower in herbal groups compared to the control group. After normalization to β-actin expression, quantitative real-time PCR analysis revealed an induction in the expression of MTR and SMS genes in the liver of both herbal treated groups. No changes were observed for the TAT, MSRB1and BHMT genes in the herbal treated groups compared to the control or the Meth40 group. In conclusion, herbal feed additives with specific plant extracts may be able to improve both growth performance and antioxidant activity of broiler chickens, phenolic content; yet, they may also support in amino acid efficient use of broiler.

Keywords: herbal extracts, lipid oxidation methionine replacer, liver gene expression

INTRODUCTION

Recently, the cost of feed raw materials has risen steeply. Prices for synthetic feed supplements, most of which are critical for livestock productivity and welfare, have quadrupled. Due to the difficulty of ensuring methionine and other essential amino acids from a single feed source, chemically synthesized sources of these amino acids are commonly employed in livestock diets. Methionine, in particular, is a sulphur-containing amino acid that serves as a substrate to all other sulphur-containing amino acids and derivative products such as taurine and cysteine (Neubauer & Landecker, 2021). The synthetic form of methionine is manufactured by petroleum in a mix of D and L variants and is typically added in conventional animal feed, whereas it is prohibited in organic farming (Willke, 2014).

Since the typical poultry diet consists of corn, other grains and soybeans, which are all relatively low in methionine, this amino acid is the most prevalent dietary limiting parameter. Poor feed conversion and stunted growth in broilers are symptoms of methionine deficiency as adequate amounts of methionine are necessary for protein synthesis (Bauchar-Thevet et al., 2008; Sekiz et al., 1975). Methionine is also necessary for the immune system's proper function and is a crucial element of feathers, hence a

methionine deficit results in poor feather growth (Sekiz et al., 1975). Recently, it was evidenced that diet deficiency in methionine affects digestibility of essential amino acids and cysteine, but not the digestibility of methionine (Fagundes et al., 2020). In addition to these economic losses from reduced feed conversion, the excess nitrogen arising from the metabolism of the non-limiting amino acids, gets rejected from the body and accumulates to the litter acting as pollutant to the environment (Onainor et al., 2021). The change in digestibility is reflected in the mRNA expression of amino acid transporters across different tissues (Fagundes et al., 2020). Because of these factors, synthetic methionine has long been the opportune solution to this problem. However, rising prices for petrol-derived chemical precursors, together with the rising demand for an organic methionine source, have prompted companies to develop herbal methionine, an organic methionine source (Hadinia et al., 2014).

Methionine is a source of two vital monomers: the methyl group and the sulphur atom. Its exact position as a methyl group contributor makes it essential for the body's energy-generating system to function properly. It is also necessary for the nucleic acid biosynthesis due to its direct involvement as a methyl group contributor and indirect role as a methyl group acceptor in folate metabolism. Likewise, as a sulphur contributor, methionine is a crucial precursor for the formation of phospholipids, taurine, carnitine, and cysteine, among other compounds. The principal structural protein in feathers, beta-keratin, is high in cysteine, and a lack of methionine, a precursor of cysteine, causes poor feathering. Furthermore, deficient birds will often turn to feather plucking in order to satisfy their methionine demand (Chen, 1993). Methionine also serves as a lipotropic factor and a precursor for the biosynthesis of glutathione, a major intermediary in the treatment of oxidative stress, in addition to its activity as a methyl group contributor and its involvement in energy metabolism. Reduced lipotropic activity causes fatty liver and belly fat accumulation in the birds, which reduces productivity. Product quality suffers as a result of disruptions in glutathione metabolism, and the birds are more vulnerable to various forms of oxidative stress (Upton et al., 2009)

Herbal feed additives are plant-based products with various properties. Methiorep™, is a phyto additive that can act as an alternative for the replacement of synthetic methionine. It contains a combination of high-protein pulses and sugars that provide substrates and intermediates for boosting the methionine metabolic pathways. Additionaly, a main consideration of the poultry industry, besides performance enhancement, is the quality of the poultry meat, especially during storage, guaranteeing a long shelf life. Lipid oxidation is a leading cause of quality deterioration in muscle foods and is well correlated with heme iron content and polyunsaturated fatty acids in meat (Rhee et al., 1996), and it is measured as rancidity. Dietary

antioxidants have been used to improve oxidative stability in meat type chickens (Christaki et al., 2012; Giannenas et al., 2016). For this reason, herbs, spices, and their extracts are examined as natural sources of antioxidants that can protect meat from oxidation and prolong storage time (Giannenas et al., 2020).

The role of liver in protein synthesis and metabolism is of paramount importance. It has been reported that diet deficiency in methionine alters the expression levels of various genes and enzyme activity of methionine oxidases (Zhang et al., 2018). Liver enzymes can modulate the transcriptional levels of several genes, which form key enzymes in biosynthesis and recycling of methionine, such as methionine synthase (5-methyl tetrahydrofolate homocysteine methyltransferase; MTR), Tyrosine aminotransferase (TAT), Spermidine synthase (SRM), methionine sulfoxide reductase (MSR) and Betaine homocysteine S-methyltransferase (BHMT). However, the effects of herbal extracts on the expression of these genes in the chicken liver have not been reported.

Therefore, an experimental trial was undertaken in order to assess the effects of an herbal additive, called MethiorepTM (Mrep), to broiler chicken diets on growth performance, breast and thigh meat chemical composition, oxidative stability during refrigerated storage and changes in genes expression levels in the liver.

MATERIALS AND METHODS

Experimental design

The trial protocol was approved by the Institutional Committee for Animal Use and Ethics of the Aristotle University Research Committee (number 99204). Throughout the trial, the birds were handled in compliance with local laws and regulations (Presidential Degree 56/2013 on harmonization of the Directive 2010/63/EU, on the protection of animals used for scientific purposes) and in accordance to the principles and guidelines for poultry welfare (NRC, 1996). The study performed in Kotopoula Barbagianni Farm, in Axos, Giannitsa, Pella (latitude 40.77°, longitude 22.45°), Greece.

In a 35 days trial, a total of 600 one-day-old (male sex) broiler chicks with average body weight 42.3 (±0.6) g were randomly allocated to 4 groups with 10 replicates (pens) of 15 chicks. All groups were housed in floor pens with rice hulls litter. The stocking density was 15 birds per m^2. During the trial, commercial breeding and management procedures were employed, natural and artificial light was provided on a basis of 23 h for the first 2 days, 16 h from day 3 to day 14, 21 h from day 15 to the slaughter day, whereas ambient temperature and humidity were controlled. All birds were vaccinated against Marek's disease after hatching, and against Newcastle disease, infectious

bronchitis and infectious bursal disease (Gumboro) during the second week of their life. Feed and drinking water were offered to all birds *ad libitum* throughout the experiment. Feed consumption and mortality within each group was recorded during the experimental period.

Control group was fed a basal diet (Table 1) based on maize and soybean meal, in mash form, according on the bird age (Starter diet 1-10 d; Grower diet 11-24 d; Finisher diet 25-35 d) which did not contain anticoccidials or antibiotics and contained 2.5 kg per ton DL-Methionine; the diets of the second group (Meth40) were similar to control diet but contained only DL – Methionine at 40% of control diet [1 kg per ton].

Table 1. Basal diets of broilers.

	Starter	**Grower**	**Finisher**
Ingredients (%)	**Days 1–14**	**Days 15–28**	**Days 29–35**
Maize	55.50	60.00	61.00
Soybean meal	35.77	30.70	28.62
Soybean oil	3.50	3.50	4.50
Palm fat	-	1.00	1.50
Calcium phosphate	1.46	1.33	1.28
Limestone (Calcium carbonate)	1.86	1.68	1.53
Salt	0.28	0.23	0.23
Sodium carbonate	0.21	0.21	0.19
L-Lysine	0.41	0.40	0.35
DL-Methionine	0.39	0.35	0.31
L-Threonine	0.22	0.21	0.15
L-Valine	0.15	0.14	0.09
Vitamin and mineral premix[1]	0.25	0.25	0.25
Total (kg)	100.00	100.00	100.00
Calculated Analysis (As fed basis)			
M. Energy[2], Kcal/kg	3000	3070	3150
Moisture, %	10.15	10.55	11.14
Crude protein, %	22.00	21.00	20.00
Crude fiber, %	2.85	2.65	2.55
Crude fat, %	4.84	6.11	6.65
Ash, %	6.12	5.65	5.58
Total Lysine, %	1.41	1.28	1.15
Total Methionine+Cystine, %	1.08	0.99	0.92
Methionine, %	0.73	0.67	0.62
Threonine, %	0.98	0.89	0.79
Tryptophan, %	0.28	0.25	0.24
Valine, %	1.10	1.02	0.92
Total NSPs[3], %	9.5	7.5	6.5
Calcium, %	0.99	0.93	0.85
Total phosphorus, %	0.71	0.65	0.62
Sodium, %	0.24	0.23	0.22
Chloride, %	0.24	0.23	0.22

[1]Supplying per kg feed: 12,000 IU vitamin A, 5000 IU vitamin D3, 30 mg vitamin E, 3 mg vitamin K, 3 mg thiamin, 7 mg riboflavin, 6 mg pyridoxine, 0.035 mg vitamin B12, 40 mg niacin, 13 mg pantothenic acid, 1.5 mg folic acid, 0.13 mg biotin, 340 mg choline chloride, 55 mg Zn, 155 mg Mn, 20mg Fe, 12 mg Cu, 0.2 mg Co, 1 mg I, 0.2 mg Se, and phytase 0.01 g, 2 M. Energy: Metabolizable Energy, 3NSPs: Non-Starch-Polysaccharides

Diet in third group contained DL-Methionine at 40% of control and the herbal feed additive MethiorepTM (Meth40+Mrep). Diet of fourth group was formulated to totally replace DL-Methionine by MethiorepTM (Mrep).

Table 2. Methiorep™ chemical composition.

Chemical analysis	**%**
Moisture	2.0-8.0
Ash	4.0-12.0
Water soluble extractive value	20.0-30.0
Methanol-soluble extractive value	9.0-15.0
Crude protein	18.0-30.0
Crude fat	1.0-4.0
Crude fiber	4.0-15.0

As mentioned above, Methiorep™ (Ayurvet, India) contains parts of plants like *Boerhavia diffusa, Azadirachta indica, Vigna mungo* and *Trigonella foenum-graecum*. The chemical composition of the product is presented in Table 2. Datasheet is presented in supplementary document 1.

Diets and herbal feed additives were analysed for their total phenolic content according to the method of Singleton et al. (1999) and expressed as gallic acid equivalents (GAE) mg/g., as determined by using the Folin-Ciocalteu assay. An aliquot (1 ml) of sample or standard solution of Gallic acid (100, 200, 300, 400, and 500μg/ml) was added to 25 ml of volumetric flask, containing 9 ml of distilled water. A blank reagent was prepared using distilled water. One ml of Folin-Ciocalteu phenol reagent (Merck, Germany) was added to the mixture and shaken. After 5 min 10 ml of 7% Na_2CO_3 solution was added to the mixture. The volume was then made up to the mark. After incubation for 90 min at room temperature, the absorbance against the reagent blank was determined at 550 nm with an UV-Visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan).

At the end of the trial (day 35) all birds were euthanatized under commercial conditions. From each replication (floor pen) 2 birds were randomly selected and further processed. Also, from these birds the breasts and thighs were removed from the carcass, were weighted and then stored for chemical analysis. All liver samples were removed, snap frozen in liquid nitrogen and stored at -80°C until analysed.

Feather score and Bedding conditions

Feathering condition was evaluated on 2 birds per pen at day 35 through a 3-point scoring system ranging from 1 to 3 (where 1 refers to clean feathers and 3 to very dirty feathers). At the same day litter moisture and litter NH_3 were analyzed as follows. From each pen, 5 litter samples of 100 g each were collected (four samples from corners and one from the center). The 5 litter samples were pooled twice and homogenized prior to dry matter analysis (2 values of litter dry matter per pen, each sample taken as a mix of all five locations). For litter dry matter analysis, the samples were weighed using precision scales, they were dried at 120°C for 6 hours and then weighed again to determine the weight difference (AHPA, 1989). Kjeldahl nitrogen (%) was determined by the Micro –Kjeldahl method (AHPA, 1989).

Meat chemical analysis

The previously collected breast and thigh meat samples were analyzed for moisture, crude protein and fat content, by near infra-red spectroscopy using a FoodScan™ Lab (FOSS, Denmark). From each sample the breast (*Pectoralis major*) or the thigh (*Biceps femoris*) meat was carefully separated from the skin and the bones, was minced (Cutter K35, Electrolux) and then 200 g of the minced meat was placed in the sample tray of the Perten DA 7250 (PERTEN, Sweden) by near infra-red spectroscopy using a transmittance mode, by the reference method 2007.04 for meat and meat products (Anderson, 2007).

Meat oxidation

Lipid oxidation of breast (*Pectoralis major*) and thigh (*Biceps femoris*) meat during refrigerated storage, was determined as malondialdehyde (MDA), using a modifed method of Ahn et al*.* (1999). The previously frozen samples were thawedp overnight at 4°C placed in a non-illuminated refrigerated cabinet, minced using a commercial food processor, wrapped in oxygen-permeable film and stored at 4 °C for a total of 6 days. On the 1st, 3rd and 6th day of refrigeration storage, subsamples were taken from each sample and processed. Absorbance was read at 532 nm against a blank sample using an UV-Visible spectrophotometre (UV-1700 PharmaSpec, Shimadzu, Japan). Results were expressed as ng of MDA per g of sample.

For the determination of protein carbonyls, the method of Patsoukis et al. (2004) was applied to meat samples of the same birds. In particular, 50 μL of 20% TCA were added to 50 μL of sample homogenate (diluted 1:2 v/v), the mixture was incubated in an ice bath and then centrifuged. The supernatant was discarded, and 2,4-dinitrophenylhydrazine (DNPH) was added in the pellet. The samples were incubated in the dark at room temperature for 1 h, and then centrifuged. The supernatant was discarded, and 1mL of 10% TCA was added, vortexed, and centrifuged. Then, the supernatant was discarded, and 1mL of ethanol-ethyl acetate (1:1 v/v) was added, vortexed, and

centrifuged. Afterwards, the supernatant was discarded, and 1 mL of 5 mol/L urea (pH 2.3) was added, vortexed, and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000 g for 3 min at 4 °C. In this assay, carbonyl formation is detected by the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) and its subsequent conversion to 2,4-dinitrophenylhydrazone (DNP-hydrazone) that is measured at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH (22x103 M^{1} cm^{-1}).

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from chicken livers stored at -80°C. Initially, the tissues were ground to a fine powder, and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion), according to the instructions provided by the manufacturer. The preliminary quantity and purity of the extracted RNA was measured at 260 and 280 nm using the BioPhotometer (Eppendorf) and RNA integrity was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. To reduce degradation, RNase inhibitor (Invitrogen) was added to each sample (1 Unit per μg of RNA) before storage at -80°C. All samples were pretreated, before reverse transcription (RT), with DNAse (Fermentas) at a concentration of 1 Unit per μg of RNA. One μg of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer instructions.

Quantitative expression analysis of the genes was performed with real-time PCR, using a LightCycler real-time PCR machine (Roche Molecular Biochemicals), as previously described (Michailidis et al., 2010), using the primers illustrated in Table 3.

Table 3. Genes, primer pair sequences (5′ to 3′), amplicon size and GenBank accession numbers, for genes amplified using sreal-time PCR analyses.

Gene	Primer pair sequence	Amplicon size	GenBank
MTR	5'-TATGCTGCTGTCAGGTCTGG-3' 5'-TGGCTACAGTCAGGGCTTCT-3'	146bp	NM_001031104.1
TAT	5'-GCTGGAGCCATGTACCTGAT-3' 5'-ACCACACGGAAGAAGTTTGG-3'	152bp	XM_414240.5
SMS	5'-CTGCGGTTGATTCTTGACCT-3' 5'-ATGTAGGAGGGAACGCACAC-3'	173bp	NM_001030803.1
MSRB1	5'-GAGGCGAAGTGTTCAAGGAC-3' 5'-ACTTGCCACAGGACACCTTT-3'	192bp	NM_001135558.2
BHMT	5'-GGTGCTTCCATTGTTGGAGT-3' 5'-CAGGTGGGCTTTCAGCTTAG-3'	108bp	XM_414685.5
β-actin	5′-CTCCCTGATGGTCAGGTCAT-3′ 5′-ATGCCAGGGTACATTGTGGT-3′	203 bp	L08165

PCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems) and 0.2 pmol of each primer in a final volume of 20 μl using as template 1/10 of the initial cDNA synthesis reaction. Gene expression levels were quantified using the β-actin as an internal standard for cDNA normalization. The cycling parameters were: incubation at 95°C for 10 min, followed by 45 cycles of incubation at 95°C for 10 sec, 56°C for 8 sec, 72°C for 8 sec, read at 60°C. For identification of the PCR products a melting curve was performed from 65 to 95°C with read every 0.2°C and 5 sec hold between reads. All the reactions were performed six times using cDNA synthesized from RNA extracted from the ovary of different birds. The threshold cycle (Ct) values of the PCRs were averaged and relative quantification of the transcript levels was performed using the comparative Ct method (Livak and Schmittgen 2001). Real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) to calculate the relative level of each mRNA in each sample and expressed as a ratio relative to β-actin housekeeping gene. The expression levels of treated groups are indicated as relative values to the control group.

Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics v. 20.0 Statistical Package (SPSS Inc., Chigaco, IL, USA). One-way analysis of variance (ANOVA) for the four groups of the experimentation was performed for all examined parameters. Also, for the meat lipid oxidation analysis a two-way ANOVA was used to estimate the effects and interaction of the group and the storage time parameters. Furthermore, a t-test was performed to estimate the significance of differences among the experimental groups. Results were expressed as the mean ± SEM. In every case the replication (floor pen) was used as the experimental unit. Tukey's multiple range test was used to distinguish the statistical difference among the mean value of each experimental group for each tested parameter. The level of significance was set at 5% (P=0.05). Values of P between 0.05 and 0.10 were considered as tendencies.

RESULTS

Performance parameters

The effects of the four different dietary interventions on broilers' performance parameters are presented in Table 4. Body weight gain was significantly lower for the broilers supplemented with the deficient diet in methionine at day 35, compared to the other three groups that had similar body weight gains during the entire experimental period (P<0,05). All treatment groups presented analogous feed intake values (P≥0,05), stating

that the different treatments did not have any unfavorable effect relative to this performance parameter.

Table 4. Effect of herbal extract dietary supplementation on broilers' performance

	Control[1]	Meth40	Meth40+ Mrep	Mrep	SEM[2]	P
BW (g)						
Day 1	42.1	42.5	42.3	42.2	0.30	0.973
Day 10	263.5	240.0	256.5	277.0	6.38	0.227
Day 24	1136.5	1085.0	1091.5	1189.5	15.98	0.070
Day 35	2088^{ab}	1960.5^{b}	2089.5^{ab}	2160^{a}	23.23	0.016
FI (g)						
Days 1-10	272.5	276.5	279.0	276.5	2.42	0.831
Days 10-24	1354.5	1367.0	1358.0	1351.5	4.97	0.728
Days 24-35	1962.5	1971.0	1967.0	2207.0	60.93	0.418
Days 1-35	3589.5	3614.5	3604.0	3835.0	60.06	0.432
FCR (feed/gain)						
Days 1-10	1.298	1.423	1.347	1.212	0.04	0.397
Days 1-24	1.503^{ab}	1.584^{a}	1.566^{a}	1.429^{b}	0.02	0.044
Days 1-35	1.761	1.889	1.769	1.819	0.03	0.530
WG (g)						
Days 1-10	221.4	197.5	214.2	234.8	6.30	0.209
Days 1-24	1094.4	1042.5	1049.2	1147.3	15.99	0.069
Days 1-35	2045.9^{ab}	1918.0^{b}	2047.2^{ab}	2117.8^{a}	23.24	0.016

[1]Control: Basal diet; Meth40: Diet contains DL –Methionine at 40% of control diet; Meth40+Mrep: Diet contains DL-Methionine at 40% of control and the herbal feed additive Methiorep™; Mrep: totally replace DL-Methionine by Methiorep™. ^{abc}values in the same line with the same superscript do not differ significantly. [2]SEM: Standard Error of Mean.

Feed conversion ratio showed insignificant differences between all groups during the intervals of day 1 to day 10 and day 1 to day 35 ($P \geq 0,05$), albeit groups Meth40 and Mrep displayed numerically lower feed conversion values corresponding to day 1 to day 24 interval ($P < 0,05$). Weight gain did not differ significantly among groups at days 1 to 10 and 1 to 24 ($P \geq 0,05$), while Mrep groups presented considerably higher weight gain values compared to Meth40 group during the period 1 to 35 days ($P < 0,05$).

Meat and liver yield and breast and thigh meat composition

Broilers' meat and liver yield plus meat chemical composition results are presented in Table 5. Methionine total substitution with the alternative product beneficially affected Mrep group that displayed significantly increased carcass yield compared to Meth40 group ($P < 0,05$). Additionally, Mrep group had also higher breast yield in contrast to Meth40 group ($P < 0,05$).

Table 5. Effect of herbal extract dietary supplementation on broilers' meat yield and chemical composition.

	Control[1]	Meth40	Meth40+ Mrep	Mrep	SEM[2]	P
Yield (g)						
Carcass	1693.4^{ab}	1590.0^{b}	1703.4^{ab}	1764.42^{a}	19.62	0.011
Breast	846.7^{ab}	795.0^{b}	851.7^{ab}	882.2^{a}	9.81	0.011
Thigh	651.3^{a}	611.5^{b}	655.2^{ab}	678.6^{a}	7.55	0.011
Liver	39.4^{b}	37.4^{b}	39.7^{ab}	43.4^{a}	5.92	0.002
Chemical composition						
Fat	9.64^{a}	8.08^{c}	9.31^{ab}	8.84^{b}	0.12	<0.001
Moisture	67.95^{b}	68.98^{a}	68.21^{ab}	68.57^{ab}	0.09	<0.001
Protein	18.87^{b}	19.30^{a}	19.04^{ab}	19.10^{ab}	0.05	0.017
Ash	3.55	3.64	3.48	3.49	0.03	0.122

[1]Control: Basal diet; Meth40: Diet contains DL –Methionine at 40% of control diet; Meth40+Mrep: Diet contains DL-Methionine at 40% of control and the herbal feed additive Methiorep™; Mrep: totally replace DL-Methionine by Methiorep™. ^{abc}values in the same line with the same superscript do not differ significantly. [2]SEM: Standard Error of Mean.

Thigh breast yield was significantly lower for Meth40 group compared to the Control and Mrep group (P<0,05). Mrep and Meth40+Mrep treated broilers exhibited significantly higher liver weight compared to Control and Meth40 treated birds (P<0,05). Meat chemical composition fluctuated among the different treatments. Meth40 group presented higher meat moisture percentage compared to the Control group (P<0,05), while Meth40+Mrep and Mrep groups did not differ drastically from either Meth40 or Control treatment groups (P≥0,05). Meat fat content was noted higher for the Control group compared to Mrep and Meth40 (P<0,05), while Mrep had higher fat content compared to Meth40 (P<0,05) and Meth40+Mrep presented similar values to both Control and Mrep groups (P≥0,05). In terms of meat protein, Meth40 treated broilers had significantly higher values compared to the Control ones (P<0,05), whereas the other two groups did not differ significantly with either of the Control or Meth40 groups (P≥0,05). Ash/Salt content was alike between all groups (P≥0,05).

Bedding moisture and N concentrations

Table 6 presents the effects of the dietary supplementation of the herbal extract on bedding moisture and N%.

Table 6. Effect of herbal extract dietary supplementation on broilers' feather score and bedding.

	Control[1]	Meth40	Meth40+ Mrep	Mrep	SEM[2]	P
Feather score	3.000	2.250	2.750	2.875	0.023	0.055
Bedding						
Moisture %	62.81	61.04	62.81	60.04	0.47	0.090
N%	1.146^{a}	1.124^{ab}	1.146^{a}	1.101^{b}	0.01	0.002

[1]Control: Basal diet; Meth40: Diet contains DL –Methionine at 40% of control diet; Meth40+Mrep: Diet contains DL-Methionine at 40% of control and the herbal feed additive Methiorep™; Mrep: totally replace DL-Methionine by Methiorep™. ^{abc}values in the same line with the same superscript do not differ significantly. [2]SEM: Standard Error of Mean.

Bedding moisture did not differ significantly among the various groups (P≥0,05). Contrarily, in terms of bedding N percentage Mrep group displayed lower values compared to Control and Meth40+Mrep groups (P<0,05), whereas Meth40 group had similar values to all groups (P≥0,05).

Determination of TBARS and Protein carbonlys

Meat TBARS values are presented in Table 7. Meth40+Mrep and Mrep treated groups demonstrated lower Tbars values during all 3 different tested periods compared to Control and Meth40, whereas Meth40+Mrep group marked the lowest values among all treatments.

Table 7. Effect of herbal extract dietary supplementation on broilers' bedding.

	Control[1]	Meth40	Meth40+ Mrep	Mrep	SEM[2]	P
TBARS						
Day 1	16.78^{a}	14.46^{a}	5.63^{b}	8.68^{b}	1.47	0.009
Day 3	17.34^{a}	18.39^{a}	6.96^{b}	8.91^{b}	1.01	0.002
Day 6	27.34^{a}	28.34^{a}	12.96^{b}	15.09^{b}	1.13	0.005
Protein Carbonyls						
Day 1	36.42^{a}	34.26^{a}	24.57^{b}	23.68^{b}	3.71	0.022

[1]Control: Basal diet; Meth40: Diet contains DL –Methionine at 40% of control diet; Meth40+Mrep: Diet contains DL-Methionine at 40% of control and the herbal feed additive Methiorep™; Mrep: totally replace DL-Methionine by Methiorep™. ^{abc}values in the same line with the same superscript do not differ significantly. [2]SEM: Standard Error of Mean.

Supplementary Meth40+Mrep and Mrep treated groups had also the lowest protein carbonyls values compared to the Control and Meth40 groups. No interactions were noted between the group and storage time effects.

Gene expression

After normalization to β-actin expression, quantitative real-time PCR analysis revealed a significant up-regulation ($P<0.05$) in the expression levels of MTR and SMS genes in the liver of both treated groups (Figure 1a, b, c, d, e). No changes ($P>0.05$) were observed for the TAT, MSRB1 and BHMT genes in the herbal groups compared to the control or the Meth40 birds.

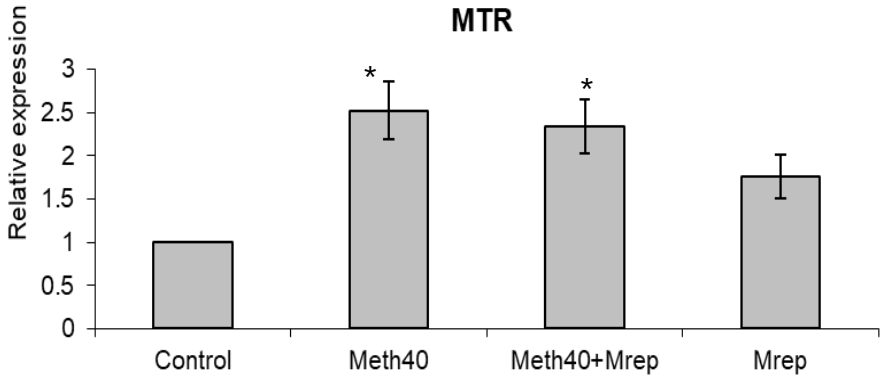

Figure 1a

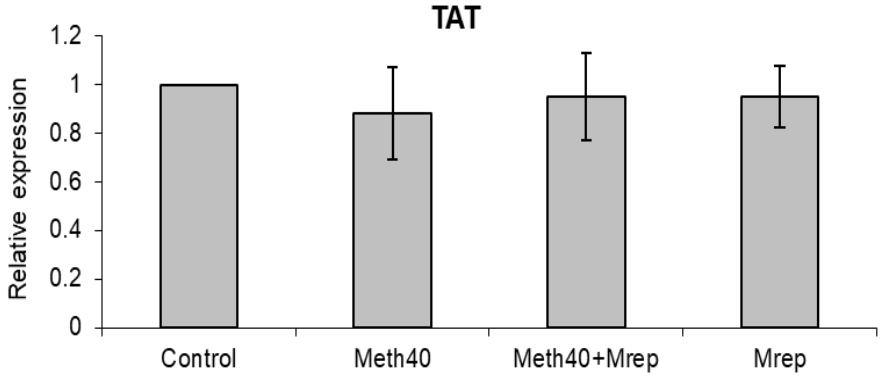

Figure 1b

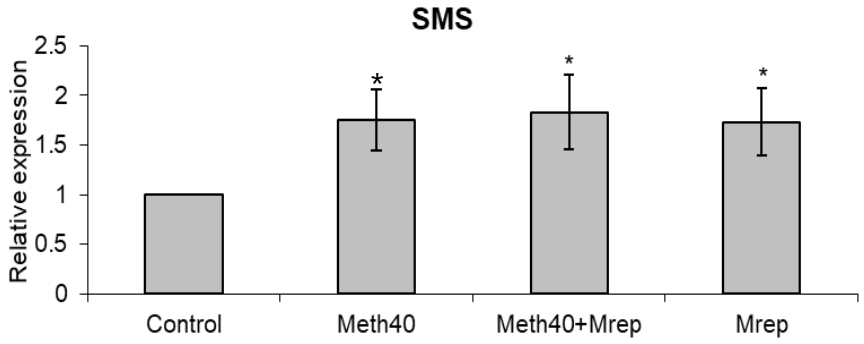

Figure 1c

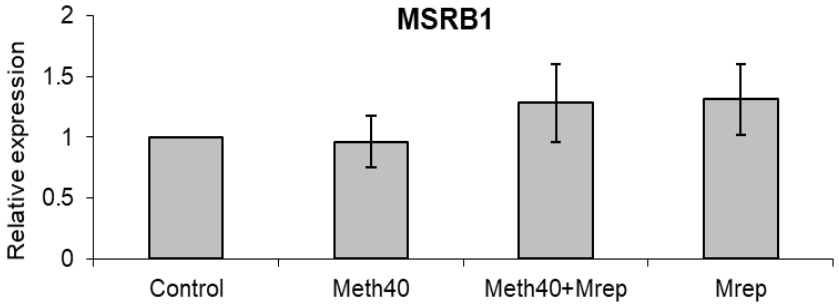

Figure 1d

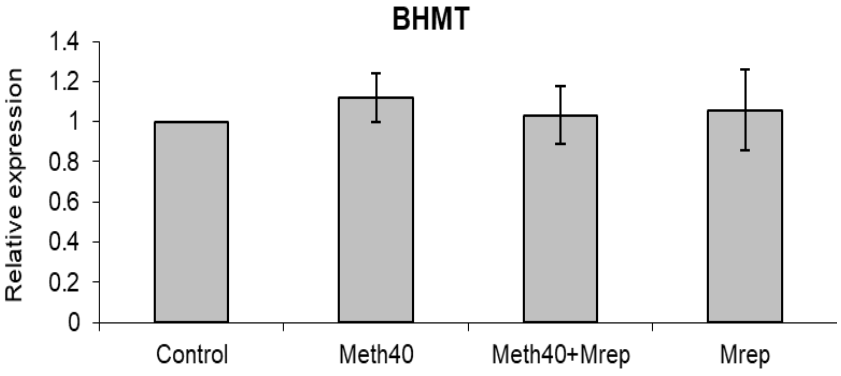

Figure 1e

Figure 1a, b, c, d, e. Changes in the expression of MTR, TAT, SMS, MSRB1 and BHMT genes in the chicken liver, following dietary supplementation with herbal feed additives. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean ± SEM (n=10). Asterisk indicates that the difference in the expression levels are significantly different ($P<0.05$).

DISCUSSION

The aim of this study was to investigate the effect of supplementation with an herbal feed additive as partial or total replacer of methionine in broiler diets. This herbal product that is a rich source of phenolic compounds, contains parts of plants such as Boerhavia diffusa, Azadirachta indica and Trigonella foenum-graecum. The effects of the herbal feed additive were assessed on the growth performance, the antioxidative capacity and the meat composition, as well as, on expressions of certain liver genes. The performance data of the present trial, confirmed that feeding a diet deficient in methionine negatively affected growth and feed efficiency. Methionine metabolic pathway follows an elaborate structure and is also linked with various other essential pathways of great importance for poultry production, such as choline, arginine, and folate, (Fagundes et al., 2020). Methionine-deficient diets result in an imbalance of nutrients, which can affect the digestibility of essential amino acids and the alteration on gene expression of amino acid transporters (Fagundes et al., 2020).

Studies evaluating feed amino acid and methionine importance have been conducted worldwide and have confirmed its main role in protein synthesis. While there is not enough data to confirm that gene regulation is fully developed in balanced or deficient diets, gene expression is affected in stress conditions, in the presence of dietary toxins and health disruptions. Furthermore, various studies have reported that animal feed contaminated with toxins can cause an array of metabolic, physiologic, and immunologic disturbances, resulting in a decrease in humoral and cellular responses and associated gene expressions in farm animals (Bryden et al., 2012; Bondy and Pestka, 2000). Despite the plethora of studies on effects of deficient diets in growth of chickens, liver gene expression of enzymes occupied in methionine cycle synthesis in farm animals and especially in poultry, is extremely scarce.

The herbal mixture named Methiorep™ may serve as a natural replacer of synthetic methionine, being cost effective and of natural origin. However, there have often been questions about its mechanism. There are conflicting results about Methiorep™ acting as a source of S-adenosyl homocysteine. The enzymes studied in the current level of expression are inclusively those that are known to hold main steps on methionine metabolism. Whether more or less methionine is required in the body, the demand for at least one of these enzymes will change. It can be shown that birds receiving Methiorep™ can have similar levels of gene expression compared to groups receiving the recommended levels of methionine in diet, whereas birds that received lower levels of methionine, could not meet the body needs of methionine synthesis even if gene expression was increased.

Amino acids also act as modulators of signaling pathways that control metabolism and cell functions (Tesseraud et al., 2011). Therefore, an

understanding of the molecular regulation of amino acid gene expression could be advantageous in optimizing dietary requirements (Fagundes et al., 2020). Dietary use of herbal extracts can be associated with mediation of toxic effects of methionine excess. Indeed, many studies have shown significantly lower levels of serum markers for liver damage viz. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) upon Methiorep™ supplementation, suggesting an absence of hepatic insult in contrast to DLM usage (Kalbande et al., 2009). Use of Methiorep™ mediated the oxidative stress associated with the use of DLM, as evident from significantly lower levels of glutathione peroxidase (GSH-Px). Furthermore, in broilers experimentally subjected to lead or cadmium toxicity, simultaneous dietary supplementation with Methiorep™ was shown to attenuate the disturbances in the activities of superoxide dismutase (SOD), reduced glutathione (GSH), catalase, Na^+/K^+ ATPase, Mg^{2+} ATPase, and cytochrome P450 (CYP450), as well as levels of protein carbonyls and thiobarbituric acid reactive substances (TBARS) (Lakshmi et al., 2011). Remarkably, broilers receiving Methiorep™ also show improved levels of additional amino acids, such as glycine, threonine, and lysine. It is known that lysine usually tends to be the second most limiting amino acid in poultry feeds (Erickson et al., 2002).

Methionine-deficient poultry diets have been proven to enhance the expression of CAT1 amino acid transporter in skeletal muscles (Fagundes et al., 2020). The transition of cationic amino acids e.g., ornithine, arginine and lysine, to different non liver tissues is regulated by CAT1. Through this process, different metabolic pathways, crucial for poultry productivity, involving amino acid inflow, protein synthesis, nitric oxide synthesis and polyamine synthesis are given as a side effect (Fernandez et al., 2003). Interestingly, CAT1 favors ornithine synthesis over nitric oxide synthesis from arginine. The acceleration of ornithine production supports spermidine and spermine synthesis, associated with essential metabolic pathway of protein biosynthesis, a supplementary mechanism of action that is supported by herbal substituted of methionine.

Quantitative real-time PCR analysis performed in this study, revealed that the mRNA abundance of MTR and SMS genes was induced following dietary supplementation with herbal feed additives**.** To our knowledge, this is the first comprehensive study on the in vivo characterization of the effects of dietary supplementation in the expression levels of these genes and demonstrates that the dietary treatment with methionine has the ability to alter the mRNA abundance of key methionine converting enzymes in the chicken liver.

Methionine is the first limiting amino acid in poultry, yet also the most toxic amino acid for chicken. Moreover, it also has a huge environmental footprint. Methiorep™ is a natural replacer of synthetic methionine and helps to overcome both of these problems. Reasonable differences were found between the estimates of the environmental impact categories of a recent

study by Anestis et al. (2020) and the estimates of related studies in the literature comparing feed additives such as toxin binders and plants extracts in the environmental impact. It has been shown that environmental footprint can be only marginally reduced when feed additives are added in piglet diets as feed additives (Anestis et al., 2020), whereas reducing the protein concentration in the diets for broilers with the addition of protease enzyme and the substitution of SBM by CGM be potential dietary strategies to lower feed cost and improve the environmental impact of broiler farming (Giannenas et al., 2017). This study showed that the group fed less methionine with the supplementation of herbal extract had satisfactory performance and meat yield and quality characteristics.

In another previous study working with two different herbal extracts as a replacement for DL-Methionine broilers achieved less weight gain (Kaur et al., 2013). The content of lipids in the liver tissue in the control diet was lower than those of the herbal supplemented groups. However, those birds showed increased immune responses, higher antibody titres against NDV and IBV vaccinations and higher level of circulatory immune complexes (Kaur et al., 2013). The effects of the herbal feed additive were assessed on the growth performance, the antioxidative capacity and the meat composition, as well as, certain liver gene expressions. The performance data of the present trial, confirmed that feeding a diet deficient in methionine negatively affected growth and feed efficiency.

Phenolic compounds are dietary constituents widely spread in plant kingdom that include thousands of compounds with different chemical structures. Due to their influence on sensorial properties (color and astringency), their analysis in foods and beverages has been developed during the last decades (Monagas et al., 2005; Fernandez-Panchon et al., 2008) to show that total phenolic content expressed in mg/g may vary between 0.24 mg/g in grape seed extracts and 147 mg/g in basil extracts. The herbal feed additive used in our study contained a high phenolic content. Methiorep™ contains extracts from plants used in traditional Indian medicine, namely Boerhavia diffusa, Azadirachta indica, Vigna mungo and Trigonella foenum-graecum.

Boerhavia diffusa belongs to the family Nyctaginaceae and is commonly known as “Punarnava”. The plant is a part of the diet of various tribes in India and Africa and it is used in Ayurveda- and traditional medicine, in general- to treat a variety of ailments, such as reproductive, urinary, respiratory, and cardiovascular disorders, inflammation, wounds and skin conditions etc. Studies of its chemical composition revealed that B. diffusa is rich in alkaloids, steroids, terpenoids, lignans and other phenolic compounds (phenolic acids, tannins, and various classes of flavonoids, isoflavonoids and rotenoids) which exert a wide array of biological activities, thus confirming many of the plant’s traditional uses (Gaur et al., 2022).

Azadirachta indica, “Neem”, (Meliaceae) is an agent of Indian and African folk medicine, revered for its numerous therapeutic applications. Almost all parts of the plant are employed for their insecticidal/larvicidal, antimicrobial (against malaria, viruses and bacterias), antidiabetic, and anticancer properties, that have been corroborated by in vitro and in vivo experiments. Other indications include skin conditions and infections, respiratory and urinary tract diseases, fever, etc. The tree’s phytochemical profile, though rich, remains to be fully elucidated: triterpenoids (such as the limonoids nimbin, nimbidin and azadiractin), steroids and saponins, diterpenoids (e.g. nimbiol) and flavonoids (azharone) are someof the bioactive compounds that have been isolated (Kharwar et al., 2020; Paul et al., 2011)

An ingredient of many South Asian dishes, Vigna mungo, or “Black gram” is a member of the Fabaceae family, with high nutritional value; the beans are rich in minerals, proteins and vitamins, dietary fibers, as well as other phytochemicals, such as steroids, phenolic acids, flavonoids and isoflavonoids, saponins, and other terpenoid compounds. Black gram pulses, roots and leaves have been traditionally used against infections, increased cholesterol and blood glucose, inflammation -both internal and external- liver diseases and nervine conditions. V. mugo extracts display interesting pharmacological actions, namely antidiabetic, antimicrobial and anthelminthic (Mowla et al., 2022; Shukla and Tyagi, 2017; Kingsley et al., 2014; Boby and Leelamma, 2003).

Trigonella foenum-graecum (“Fenugreek”) is another member of the Fabaceae family, consumed either dry, as a spice, or fresh, as a vegetable. Fenugreek has been traditionally used as a bitter agent, to increase appetite and stimulate digestion. It has also been employed to stimulate milk production both in women and cattle. Many studies have proved fenugreek’s activity in lowering blood glucose and cholesterol levels Estrogenic, antioxidant and anti-cancer activities have also been reported. Essential oil (with anethole being the main ingredient), alkaloids (trigonelline), steroidal saponins (such as diosgenin) and flavonoids (mostly quercetin glycosides) are the most abundant classes of secondary metabolites found in fenugreek extracts (Knott et al., 2017; Belguith-Hadriche et al., 2013; Srinivasan, 2006). Therefore, a likely interpretation of our findings is that the increased dietary phenolic content exerted its biological properties and influenced positively the chicken performance and the meat lipid oxidative stability. Lately, there is an increased research interest on the utilization of natural bioactive substances in farm animal nutrition (Giannenas et al., 2020).

The second objective of our study was to investigate whether the sustained consumption of different herbal mixtures would affect the antioxidant status of chicken breast and thigh meat. A sensitive marker to measure small changes in meat lipid peroxidation, is the formation of malondialdehyde: unsaturated lipids, after ingestion, may be prone to

oxidation and subsequent formation of aldehydic toxic molecules acting as reactive oxygen compounds. Protein oxidation is leading to protein carbonyls formation. In our study, lipid oxidation test has shown that the herbal feed additive protected chicken meat compared to controls. TBARS high values are in a positive correlation with other determinants of warmed-over flavor, such as sensory evaluation scores and pentanal and hexanal formation. Differences in lipid oxidation were also noted among breast and thigh chicken meat, possibly due to their different fat content (Rhee et al., 1996; Katsanidis et al., 2003). Our results provide evidence of reduced meat oxidation in the groups supplemented with the herbal extract. Several studies have shown that oriental herbal extracts can provide potent antioxidant properties (Fernandez-Panchon et al., 2008; Waskar et al., 2011), however, it should be noted that the relationship between the phenolic structure and their bioactive functions is not fully clarified (Gessner et al., 2017) and further research is needed in order for the phenolic content in chicken tissues to be qualified and quantified. Our findings are in agreement with previous publications working with similar oriental plants that also exhibited dietary potent antioxidant protection (Giannenas et al., 2018; Giannenas et al., 2019). However, we found severe changes in amino acid composition of egg albumen containing higher percentages of amino acids containing aromatic rings (Giannenas et. al., 2021)

CONCLUSION

In conclusion, the results presented in this paper suggest that a specific herbal feed additive may be able to improve both growth performance and antioxidant activity of broiler chickens. Collectively, the data provided in this study reveal that certain members of the chicken methionine converting enzymes are regulated in the chicken liver, following treatments with less methionine or diets supplemented with herbal methionine precursors. These novel findings provide strong evidence to suggest that methionine deficiency is accompanied with growth decline and has a suppressive effect on protein synthesis, through the alteration in the expression levels of key genes involved in the methionine synthesis cycle and lower body weight gain. Further experiments are necessary in order to determine enzyme activation and signalling patterns following treatments with deficient and abundant amino acids in the chicken diet. A clear understanding of the effects of methionine deficiency and alternative herbal mixtures in poultry diets may contribute to the reduction of farm household economic losses, reduced environmental footprint, and increased broiler performance both in organic and conventional breeding.

CONFLICT OF INTEREST

This study was funded by M/s Ayurvet Limited and BG was employed by M/s Ayurvet Limited during this study. M/s Ayurvet Limited manufactures and markets Methiorep commercially. However, the funding support and affiliation did not influence the outcomes of the study in any manner.

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