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Research Article

Assessing the relative effectiveness of synthetic and herbal choline supplements on growth performance, carcass quality and gene expression studies in broilers

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Abstract

Broilers on high-energy diets face metabolic challenges, including fatty liver syndrome and related complications. Choline, crucial for acetylcholine synthesis and liver fat metabolism, influences nutrient assimilation and energy metabolism, with deficiency causing growth issues and health complications. This study evaluated the potential of herbal choline supplements derived from specific plants as an alternative to synthetic choline. A total of 200 day-old Vencobb broiler chicks were randomly allocated into four groups, each comprising 50 birds. Each group was further divided into five replicates, with 10 chicks per replicate. The experimental diets were organized as follows: a control group (T0) fed a basal diet; T1, supplemented with synthetic choline chloride (600 g/tonne) and biotin (150 g/tonne); T2, supplemented with Repchol (500 g/tonne); and T3, supplemented with a local herbal choline product, Brand A (1000 g/tonne). Results indicated herbal choline supplements surpassed synthetic choline chloride effects. Improved growth and performance, particularly in T2, were observed. Gene expression indicated herbal choline's distinct molecular pathway compared to synthetic choline chloride, as it did not significantly ($P>0.05$) influence *PEPCK* and *FAS* genes. Carcass quality parameters revealed positive impacts in T2, including live weight, dressed weight, breast meat yield, and abdominal fat reduction. Carcass traits, as a percentage of live weight, significantly ($P<0.05$) improved in herbal supplement groups. The study highlights herbal choline's potential as a viable synthetic choline substitute in broiler diets. Their enhanced impact on growth, overall performance, and carcass quality supports the exploration of natural substitutes, encourages sustainable practices, and addresses the challenges associated with using synthetic additives in poultry diets.

Keywords: Herbal Choline, Choline supplements, Gene expression, Performance

Highlights

- Improved growth: Herbal choline was found to be equal to or more effective than synthetic choline in improving broiler performance.
- Positive carcass quality: Broilers fed herbal choline showed higher live weight and better yields.
- Distinct molecular pathway: Herbal choline affected a unique pathway, not altering *PEPCK* or *FAS* genes.
- Superior performance: Herbal choline outperformed control and synthetic groups in all parameters.
- Sustainable alternative: Herbal choline serves as a natural and efficient alternative to synthetic choline chloride.

INTRODUCTION

Poultry production stands as a cornerstone in the global agricultural industry, providing a crucial source of high-quality protein through broiler chickens for human consumption. Achieving optimal growth, health, and performance in broilers necessitates meticulous attention to their dietary requirements. Among these essential nutrients, choline emerges as a key player in various physiological functions, including cell membrane integrity, neurotransmitter synthesis, and lipid metabolism. As the poultry industry evolves, researchers and producers are exploring innovative approaches to enhance broiler performance and well-being. The integration of herbal choline sources into diets has surfaced as a compelling topic, aligning with the growing interest in sustainable and natural solutions for livestock production.

Choline, an essential element for the synthesis of acetylcholine, a neurotransmitter critical for transmitting nerve impulses which holds a key role in broiler physiology. Additionally, choline is integral to liver fat metabolism, preventing abnormal fat accumulation by facilitating transport and enhancing fatty acid utilization (Xu *et al.*, 2010). Classified as a B-complex vitamin, choline's significance extends to optimizing energy metabolism, regulating fatty liver syndrome, and reducing body fat (Huang *et al.*, 2007).

Choline deficiency leads to fatty liver syndrome and

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perosis in birds, and disrupts liver fat mobilization due to insufficient carrier lipoproteins (Pompeu *et al.*, 2011). Fast-growing commercial broiler chicks on high-energy diets with limited exercise commonly experience fatty liver syndrome, due to inadequate choline or biotin in their feed. Young chicks, unable to produce choline in the liver, are more prone to this disease than adults. Supplementing choline in high-energy broiler diets has been shown to enhance feed consumption, body weight gain, and feed conversion efficiency (Emmert and Baker, 1997). Choline deficiency also causes growth retardation and bone deformities in fast-growing broiler strains (Igwe *et al.*, 2015). Affected birds often exhibit tibial-tarsal rotation, tendon dislocation, and leg joint hypertrophy; in adults, ascites and cirrhosis may occur (Selvam *et al.*, 2018).

Choline, traditionally supplied through synthetic sources like choline chloride, has sparked recent interest in its herbal counterparts. Derived from plant-based materials, herbal choline offers a natural alternative with potential bioactive compounds that could contribute to improved broiler health outcomes (Kidd, 2007). This paper seeks to present a detailed analysis of choline usage, including both synthetic and herbal variants, in broiler nutrition. The nutritional significance of choline, its roles in broiler physiology, and emerging evidence on the effectiveness of herbal choline sources will be explored. Understanding the implications of including herbal choline in broiler diets becomes imperative as the poultry industry adapts to the changing agricultural landscape.

Research reveals that the partial replacement of synthetic feed additives like methionine with herbal alternatives promote greater body weight gain, improved feed conversion efficiency, and a higher dressing percentage and a less stress in broilers (Kumar *et al.*, 2018). Herbal compounds containing high levels of choline are emerging as alternatives to choline chloride for broiler diets. These products are rich in phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine, offering higher bioavailability due to better gut-receptor affinity (Calderano *et al.*, 2015; Farina *et al.*, 2017). Herbal choline's components, including phosphatidyl inositol and phosphatidylserine, are crucial for phosphatidylcholine metabolism, enzymatic control, and growth response (Khosravinia *et al.*, 2015). Herbal choline improves energy metabolism and nutrient utilization, preventing energy from converting into lipids and fatty liver syndrome (Koujalagi *et al.*, 2018). Additionally, these vegetable substances are less hygroscopic, preserving the integrity of other nutrients, such as water-soluble vitamins.

Studies indicate that adding herbal choline products can mitigate the adverse metabolic effects of high-energy diets in broiler chickens. Unlike synthetic choline, herbal choline does not convert to trimethylamine in the gut, avoiding harmful chemicals. These herbal additives provide multiple benefits compared to synthetic choline chloride. They are rich in choline, making them practical substitutes for choline supplementation in broiler diets (Hoyles *et al.*, 2018). Additionally, herbal choline has lower hygroscopic properties than synthetic choline chloride, ensuring stability and preventing moisture absorption from the atmosphere, which helps maintain feed integrity. The performance of broilers fed synthetic choline chloride and those supplemented with herbal choline alterations have been compared in several researches (Martínez *et al.*, 2021). Numerous studies have been conducted on the effects of choline supplementation on broiler growth. Many studies have been conducted on using synthetic and herbal choline additions in broiler diets (D'Souza and Selvam, 2022). Research has demonstrated that choline supplements, whether synthetic or herbal, can enhance broiler performance in terms of growth rate, feed efficiency, and nutrient utilization (Makała, 2021).

Hence, this study primarily aims to evaluate the production performance of herbal choline as a substitute for synthetic choline chloride in broiler diets, with a specific focus on performance up to 42 days of age. It is hypothesized that broilers receiving herbal choline will exhibit superior growth performance, enhanced carcass quality, and differential gene expression related to metabolism and growth compared to those receiving synthetic choline chloride, due to the greater bioavailability and favourable metabolic profile of herbal choline sources.

MATERIALS AND METHODS

Study location

The trial was conducted at the Poultry Experimental Station located within the Livestock Farm Complex of NTR College of Veterinary Science in Gannavaram, positioned at 16°31′ 44″ N latitude and 80°47′ 45″ E longitude, with an elevation of 82 feet (25 meters) above mean sea level. The experimental period extended from January 20, 2020, to March 3, 2020.

Pre-experimental preparations

The study was conducted at the Department of Animal Nutrition and the Livestock Farm Complex of NTR College of Veterinary Science, Gannavaram. Laboratory analyses were conducted at the Departments of Animal Nutrition and Veterinary Biochemistry within the college, covering several parameters. Feed ingredients such as maize, soybean meal, and vegetable oil for the preparation of experimental diets were obtained from the local market. The chemical analysis

Table 1. Experimental design

Sl. No.	Group	Dose rate	No. of Birds
1	Control (T0): Basal diet	—	50
2	Treatment (T1): Basal diet supplemented with synthetic choline + biotin	Synthetic choline chloride @ 600 g/tonne of feed + biotin @ 150 g/tonne of feed	50
3	Treatment (T2): Basal diet supplemented with Repchol**	Repchol @ 500 g/tonne of feed	50
4	Treatment (T3): Basal diet supplemented with Brand A*	Brand A (local variety)* @ 1000 g/tonne of feed	50

Repchol**- Poultry feed supplement is a combination of vitamins, minerals, amino acids, and other essential nutrients, including 500 mg/kg of choline, required for the growth and development of poultry birds.

*A—LIPO CARE advanced- by Varsha group- Lipocare (non-herbal) is a combination of lecithin (PC), emulsifiers and lipase promoting factors (LPF) provide safe and better replacement to choline chloride.

of feed ingredients was carried out to determine their proximate composition (BIS, 2007). Basal diets (control) for broilers were formulated for both the starter and finisher phases according to the poultry nutrient requirements (BIS, 2007).

Preparation of phytogenic mixture

The natural growth promoter was provided by M/s. Ayurvet Pvt Ltd., Baddi, H.P., India, and other feed additives were procured locally.

Experimental animals and management

The birds received immunization for Marek's disease at the hatchery, Newcastle disease (ND) with Lasota vaccine on days 7 and 28 of age, and IBD vaccine on days 14 of age through the ocular route.

Two hundred (200) day-old Vencobb broilers were obtained and divided randomly into four equal groups, each having five replicates of ten chicks. All groups, including the control group (T0), were provided with a standard basal diet (ME 3102 and 3200 Kcal/kg; CP% 22.32 and 20.28 in starter and finisher phases, respectively) with no added choline supplement. Groups T1, T2, and T3, however, received additional supplements: synthetic choline chloride at 600 g plus biotin at 150 g per tonne of feed, Repchol® (from M/s. Ayurvet Ltd., India) at 500 g per tonne, and Brand A* at 1000 g per tonne of feed, respectively. The birds were raised on deep litter until six weeks of age with free access to feed and water. Nutrient content in the experimental diets was determined using standard proximate analysis techniques (AOAC, 1996). The experimental design is presented in Table 1, while Table 2 details the ingredient and chemical

Table 2. Ingredient and chemical composition of the starter and finisher diets (%) of basal diet for different growth phases

Feed ingredient	Starter diet (kg)	Finisher diet (kg)
Ingredient composition		
Maize	53	56.20
Vegetable oil	3.85	5.45
DORB	2.30	2.30
Soybean meal	37	32
DCP	1.73	1.67
Shell grit	1.06	1.30
Trace Min. Mix.	0.20	0.20
Salt	0.30	0.30
Lysine	0.30	0.30
DL-Methionine	0.18	0.17
Vit AB2D3	0.05	0.05
Choline chloride	0	0
Coccidiostat	0.01	0.05
Antibiotic	0.02	0.01
Sub Total	100	100
Chemical composition on DMB (%)		
Dry matter	91.43	91.24
Organic matter	92.07	93.57
Crude protein	22.32	20.28
Ether extract	6.16	4.59
Crude fibre	4.65	5.67
NFE	58.94	63.03
Total ash	7.93	6.43
AIA	1.45	1.54
Calcium (%)	1.27	1.27
Phosphorus (%)	0.84	0.84
ME* Kcal/kg	**3102**	**3200**

*Trace mineral mixture: - Each kg contains: Pre-mix contains vitamin AB2D3 (0.05 kg): Vit-A 82,500 IU, Vit-B2 50 mg, Vit-D3 12,000 IU, Vit-K 10 mg; Trace minerals (0.2 kg): manganese sulphate 55000 mg, ferrous sulphate 50000 mg, zinc sulphate 50000 mg, cobalt sulphate 500 mg, copper sulphate 3000 mg, potassium iodide 3000 mg, sodium selenite 500 mg. Coccidiostat (0.02-0.03 kg) and antibiotic (Chemoxy 200-oxy tetracycline 0.01%) were added.

composition (%) of the starter and finisher diets for each growth phase.

Data collection

Growth and performance parameters: The data collection process involved monitoring growth and performance parameters, where daily and weekly feed consumption were estimated, and feed conversion ratio (FCR) was calculated as the ratio of total feed consumed to the gain in body weight. Body weight gain was assessed through weekly measurements of individual bird weight, starting from day-old age.

Gene expression studies: Gene expression was studied with liver tissue among treatment groups, particularly in genes related to nutrient metabolism. Two candidate genes were chosen for gene expression differences among different treatment groups. First, the phosphoenolpyruvate carboxykinase gene (*PEPCK*), which is known to be involved in gluconeogenesis; second, the fatty acid synthase gene (*FAS*), which is known to be involved in fatty acid synthesis, in order to understand the role of choline in nutrient metabolism. The beta actin gene is used for normalization of the gene expression.

Total RNA was isolated using TRIzol method. First strand cDNA was synthesized using Prime script RT-PCR kit (TAKARA bio). Gene expression was analyzed using a SYBR Green based real-time PCR assay. Chicken beta actin gene was used to normalize the gene expression. The primers used in the study are presented in Table 3.

Table 3. Primers used in the study

ACTB_F	GCCAACAGAGAGAAGATGACAC
ACTB_R	GTAACACCATCACCAGAGTCCA
*PEPCK*_F	GAGAGCCTGCCTCCACAA
*PEPCK*_R	CGACCCAGCTGGCTACTT
*FAS*_F	CAATGGACTTCATGCCTCGGT
*FAS*_R	GCTGGGTACTGGAAGACAAACA

Relative expression of each treatment in comparison with T1 is considered as fold difference in expression. Total RNA was isolated, and gene expression was analyzed using real-time PCR assays, with the chicken beta-actin gene used as a reference for normalization.

Carcass quality parameters: At the end of the experiment, the birds for slaughter (2 birds/per replicate) from the respective treatments were randomly chosen, weighed, slaughtered, and live weight (g), dressed weight(g), liver(g), heart(g), gizzard(g), spleen(g), kidney weight(g), breast meat yield (g), abdominal fat(g), weight of drumstick, abdominal fat per cent, drum-stick per cent, breast meat per cent, giblet per cent, pH and dressing per cent were estimated.

Statistical Analysis

The collected data was statistically analyzed using a Complete Randomized Design, and the treatment averages were compared using Critical Differences (Snedecor and Cochran, 1994).

RESULTS

Feed intake, body weight, and feed efficiency of broilers: In this study, the feed consumption of experimental broiler chicks was systematically recorded at weekly intervals throughout the six-week experiment. The data obtained under various experimental treatments are presented in Table 4.

The data in the table encompasses key indicators of feed efficiency, such as feed intake (FI), body weight gain (g), and feed conversion ratio (FCR) over six weeks. Notably, the T1 group exhibited significantly lower FI compared to the control and other treatment groups (P<0.05). Conversely, the T2 group demonstrated a significantly higher average body weight gain and a more favourable FCR (P<0.05) than other treatment groups, highlighting its superior performance.

Table 4. Effect of treatments on weekly body weight gain (g), feed intake (g) and FCR in broilers

Treatment	Body weight gain (g)	FI (g)	FCR
T0	$1855.48^{c}\pm24.54$	$3419.52^{b}\pm32.80$	$1.84^{a}\pm0.05$
T1	$1930.40^{b}\pm29.48$	$3407.88^{c}\pm29.51$	$1.77^{b}\pm0.07$
T2	$1971.54^{a}\pm31.52$	$3429.56^{a}\pm31.87$	$1.73^{c}\pm0.04$
T3	$1925.30^{b}\pm32.61$	$3419.70^{b}\pm28.91$	$1.78^{b}\pm0.02$
P-value	0.000	0.001	0.00
n	50	50	50

^{abc} Values in columns bearing different superscripts differ significantly (P<0.05)

Carcass characteristics: The data on carcass characteristics (Table 5) showed significant difference (P<0.001) in all the parameters except for abdominal fat.

Table 5. Effect of treatments on carcass traits

Treatment	Liveweight (g)	Dressed Wt. (g)	Giblet weight in (g)			Spleen (g) ^{NS}	Kidney (g) ^{NS}	Breast meat yield (g)	Abd. fat (g)
			Liver (g)	Heart (g)	Gizzard (g)				
0	1928.20^{c} ±2.31	1395.19^{b} ±14.09	42.90^{a} ±0.48	9.40^{a}± 0.34	44.30^{a} ±0.82	2.50 ±0.17	2.80 ±0.13	350.50^{c} ±2.22	76.80 ±1.04
1	2003.10^{b} ±23.16	1444.91^{a} ±13.35	40.30^{b} ±0.87	9.00^{a} ±0.26	29.60^{b} ±0.88	2.10 ±0.28	3.02 ±1	384.10^{a} ±7.08	78.80 ±1.20
2	1969.10 ^{a} ±1.12	1451.76^{a} ±4.89	35.50^{c} ±0.58	10.40^{b} ±0.34	43.60^{a} ±0.70	2.50 ±0.17	3.10 ±0.41	377.30^{a} ±3	80.50 ±1.17
3	1929.10 ^{c} ±3.37	1398.56^{b} ±7.96	39.80^{b} ±0.98	11.70^{a} ±0.33	43.10^{a} ±0.48	2.30 ±0.15	3.20 ±0.29	364.60^{b} ±2.68	79.20 ±1.17
P Value	.000	.000	.000	.000	.000	.430	.772	.000	.167
N	10	10	10	10	10	10	10	10	10

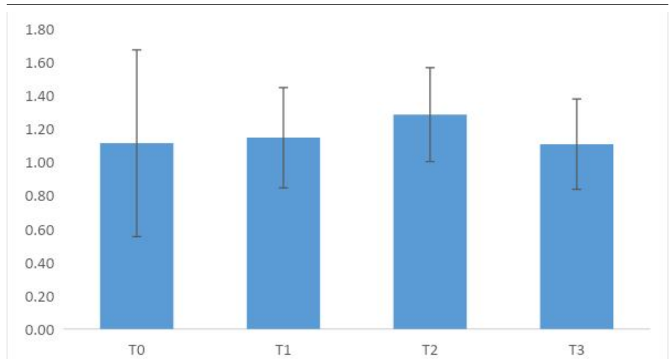
^{abcd} Values in columns bearing different superscripts differ significantly (P<0.05)

In the present experimental study, the *PEPCK* gene and *FAS* gene didn't show any differences in expression between different groups. Since, *PEPCK* gene is related to glycolysis, the choline chloride action may not be influencing this gene significantly.

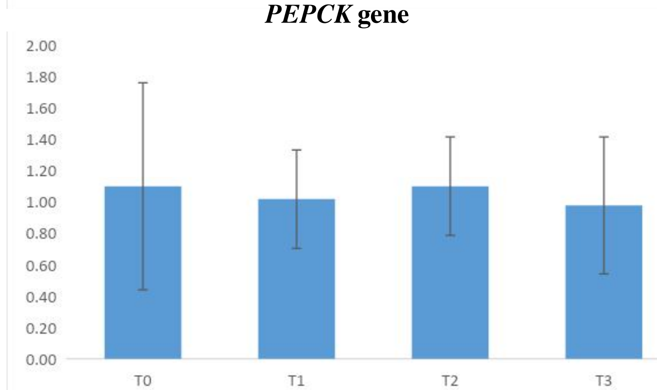
The mean fold differences in the expression of the *PEPCK* gene and the *FAS* gene in different treatment groups are presented in Table 6 and represented in Graph 1.

Table 6. Effect of treatments on gene expression parameters (fold change) in broilers

Gene	T0	T1	T2	T3
PEPCK	1.11±0.56	1.14±0.30	1.28±0.28	1.11±0.27
FAS	1.10±0.66	1.02±0.31	1.10±0.31	0.97±0.44



***PEPCK* gene**



***FAS* gene**

Graph 1. Gene expression differences in different treatment groups in *PEPCK* and *FAS* gene in relation to treatment group

DISCUSSION

Improved feed intake and body weight gain and feed conversion ratio (FCR): Birds receiving diets with herbal choline at 0.500 kg/tonne of feed (T2) exhibited significantly higher cumulative feed consumption compared to the control group and groups supplemented with synthetic choline (T1). The slight improvement in feed intake observed in herbal choline incorporated groups could be attributed to the plant ingredients containing broad spectrum of vitamins, acids, and alkaloids, which are known to increase bile flow and improve feed intake.

The findings of the present study align with those of Khose *et al.* (2019), who observed significant improvement in live body weights with dietary supplementation of herbal choline at 0.250, 0.350, and 0.500 kg/tonne of feed. Muthukumarasamy *et al.* (2004) also reported a significant (P<0.05) improvement in body weights of broilers with supplementation of Biocholine at 0.75 kg/tonne. Conversely, Calderano *et al.* (2015) observed no significant differences in live body weights with dietary supplementation of vegetal sources of choline at 100, 150, 200, 250, and 300 mg/kg in male broiler birds. Filho Demattê *et al.* (2015) also reported no significant difference in body weights of broiler birds with dietary supplementation of natural and alternative sources of methionine and choline. Similarly, Kathirvelan *et al.* (2013) found no significant difference in body weights in broilers with natural choline supplementation at 1 kg/tonne of feed.

Regarding FCR, our study demonstrated a significantly improved ($P<0.001$) feed conversion ratio in broilers from the supplemented groups T2 (1.73) and T1 (1.77) compared to the control group (1.84). These findings are in line with those of Khose *et al.* (2019), who observed significant ($P<0.05$) improvement in FCR with dietary supplementation of herbal choline at 0.250, 0.350, and 0.500 kg/tonne of broiler feed. The current study suggests that the lipotropic activities in Biocholine and choline chloride may enhance energy utilization, resulting in increased weight gain with no change in feed intake and slight improvements in FCR. Moreover, in line with earlier research (Khosravinia *et al.*, 2015; Khose *et al.*, 2019), herbal choline supplementation significantly improves feed efficiency and FCR in broilers. Rodelas *et al.* (2011) also reported improved FCR with dietary supplementation of Biocholine in broiler feed. Similarly, Khosravinia *et al.* (2015) observed improvement in feed conversion ratio with dietary supplementation of Biocholine at 1 g/kg in broiler diets. In contrast, the study differs from the observations of Lopez and Leeson (1995), which emphasized the importance of dietary lysine levels for broilers without any mention of choline. Rodelas *et al.* (2011) also demonstrated that, aside from overall feed consumption, feed efficiency did not significantly differ among dietary treatments supplemented with choline chloride or Biocholine, with or without a combination of herbal vitamins E and C. Another study by Kathirvelan *et al.* (2013) found no significant effect on feed conversion ratio in broilers with natural choline supplementation at 1 kg/tonne of feed. Additionally, Dias *et al.* (2022) did not observe any significant difference in feed conversion ratio in broilers with supplementation of plant-sourced choline as a replacement for 25%, 50%, and 100% of choline chloride.

Carcass characteristics: Current study using herbal choline have reported significant differences ($P<0.05$) in carcass weight and dressing percentage compared to studies using choline chloride in the diet. These findings suggest that supplementing herbal choline at a rate of 0.500 kg/tonne in the feed improves broiler carcass traits. In contrast to the findings of Khosravinia *et al.* (2015), where the inclusion of lipotropic agents in the diet had no effect on carcass yield, our study shows a positive impact. Similarly, Igwe *et al.* (2015) reported no significant differences ($P>0.05$) in carcass weight or dressing percentage between birds fed herbal choline and those receiving synthetic choline chloride.

Fatty acid synthase is a key enzyme in lipogenesis. Choline is a lipotropic agent and is expected to increase lipogenesis. However, the similarities in expression of *FAS* gene in the three groups probably suggest that choline may not be influencing the *FAS* gene expression and perhaps uses a different molecular pathway in exhibiting its function.

The *PEPCK* gene, also known as phosphoenolpyruvate carboxy kinase, is involved in the process of glycolysis. Glycolysis is a metabolic pathway that converts glucose into pyruvate, which can then be used to produce energy. The expression of *PEPCK* gene did not show any differences between the different groups in this study. This suggests that choline chloride, a compound that is expected to increase lipogenesis, may not be influencing the expression of this gene.

Gene expression: The *FAS* gene, also known as fatty acid synthase, is a key enzyme in the process of lipogenesis. Lipogenesis is the synthesis of fatty acids from acetyl-CoA and malonyl-CoA, and it plays a crucial role in the production of lipids. *FAS* gene expression also did not show any differences between the different groups in this study (Table 6). This suggests that choline may not be influencing the expression of *FAS* gene and may instead be using a different molecular pathway to exhibit its effects.

Choline is a lipotropic agent, which means it helps to metabolize and transport fats. It is expected to increase lipogenesis and promote the synthesis of fatty acids. However, the similarities in the expression of the *FAS* gene in the three groups suggest that choline may not be directly influencing the *FAS* gene expression. Choline may be using a different molecular pathway to exert its effects on lipogenesis. To date, limited research has been done to study the molecular pathways of using choline and herbal choline in poultry diets.

The study findings highlight the positive impact of incorporating herbal choline (Repchol) at 500 g/tonne in broiler diets, serving as a beneficial source of choline chloride. This inclusion proves advantageous by increasing live body weight, weight gain, and overall broiler performance, while also improving feed consumption and conversion efficiency. Notably, the herbal choline (Repchol) demonstrates no adverse effects on carcass characteristics in broilers. Moreover, the herbal choline analogs outperform both the control group and synthetic choline (T1) groups across all evaluated parameters. Although there is no significant variation in gene expression among treatment groups for *PEPCK* and *FAS* genes, there is a consistent trend of overexpression in groups with herbal feed additives compared to T1 and T0 groups. Overall, these findings highlight the potential of herbal choline as a valuable dietary supplement in optimizing broiler performance and health.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: AKC, MM: Involved in investigation, data generation, and preparing original draft; ST: Engaged in conceptualization, data curation, supervision and final editing; NSM: Involved in statistical analyses; BG: Involved in methodology and editing.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

Ethical statement: This study adheres to the principles of ethical treatment and experimentation on animals. All experimental procedures performed were approved by the Institutional Animal Ethics Committee (IAEC) under reference number 15/IAEC/NTR CVSc/2019, dated 14.12.2019. The research was conducted in compliance with the general guidelines of Good Clinical Practice (GCP) as outlined in the VICH Standard for Good Clinical Practice (V). The utmost care was taken to minimize stress and discomfort to the animals throughout the study.

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