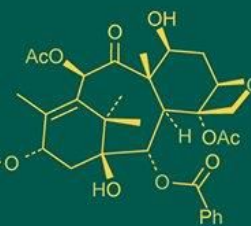
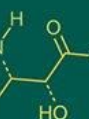
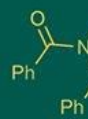


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Efficacy evaluation of phytogenic choline formulation and its comparison with synthetic choline chloride in choline deficiency model in broiler chicken on different production parameters, carcass traits, serum biochemistry and liver histopathology

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Abstract

This study assessed the efficacy of a novel Phytogenic formulation, natural herbal choline (NHC) in a broiler choline deficiency model (CDM) induced by replacing soybean meal (SBM) with corn gluten meal (CGM) and soya protein isolate (SPI). A total of 896 one-day-old Ross 308 broiler chicks were allocated to three experiments. Experiment 1 established the CDM using 0%, 25%, 40%, and 60% SBM replacement. Experiment 2 compared NHC (350 and 700 g/ton) against SCC (1000 g/ton) in the CDM (40% CGM & SPI). Experiment 3 determined the dose-response of NHC (100, 200, and 250 g/ton) against SCC (450 g/ton). Body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), and mortality were evaluated at 21 and 42 days. Serum AST and ALT, breast muscle lipids, and carcass traits were evaluated at 42 days. Replacement of SBM with CGM & SPI induced a significant ($p < 0.01$), dose-dependent decline in BWG and FCR at both 21 and 42 days. The 40% CGM replacement was selected as the optimal CDM. NHC at 250 g/ton showed similar performance to SCC at 1000 g/ton in BWG and FCR in CDM. NHC at 250 g/ton significantly outperformed SCC (450 g/ton), improving final BWG and FCR. The CDM elevated serum AST and ALT at day 42, which were more effectively normalized by NHC (250 g/ton) than by SCC. NHC at 250 g/ton also showed superior reduction ($p < 0.05$) in triglyceride and cholesterol content in breast muscle compared to SCC. Carcass yield was slightly improved in NHC groups. A choline deficiency model was successfully established using 40% SBM replacement. The polyherbal formulation, particularly at 250 g/ton, demonstrated efficacy similar to synthetic choline chloride in enhancing growth performance, liver function, and muscle lipid profile, presenting a superior natural alternative for poultry production.

Keywords: Broiler, choline deficiency, corn gluten meal, soya protein isolate, dose-response

1. Introduction

Choline is a vital micronutrient that plays a crucial role in maintaining cellular and organismal homeostasis. As a fundamental element of phospholipids and sphingolipids, it is essential for the structure and functionality of membranes. Furthermore, choline is a precursor to acetylcholine, an essential neurotransmitter, and to betaine, a methyl donor that plays an important role in epigenetic regulation (Wauben and Wainwright, 1999) [25]. Choline, a quaternary ethanolamine primarily found in the form of phospholipids, serves various biological functions in poultry. It is essential for the building and preservation of cell membranes and organelles like mitochondria and microsomes, as well as for the development of the cartilage matrix in bones. (Arele *et al.*, 2015) [12]. Although choline can be produced *de novo*, the endogenous synthesis in poultry is frequently inadequate, resulting in deficiency symptoms like growth retardation and perosis in young chicks (NRC, 1994) [20]. Significantly, dietary methionine or methyl donors alone may not sufficiently fulfill choline needs, since avian species have a limited ability to perform the initial step in choline biosynthesis—the methylation of aminoethanol to methyl aminoethanol (Dilger *et al.*, 2007) [8].

Additionally, incorporating exogenous choline, such as synthetic choline chloride (SCC) into commercial feed formulations is vital for sustaining normal physiological functions and enhancing productivity. Approximately 70% of choline chloride is not absorbed in the gastrointestinal tract; rather, it is converted by intestinal bacteria into trimethylamine (TMA), which is a toxic substance (Hoyles *et al.*, 2018) [13]. One notable limitation of choline chloride is its tendency to absorb moisture due to its hygroscopic nature, which causes clumping in feed premixes. This characteristic results in the instability of vitamins (particularly vitamins A, D₃, and E) and various micronutrients when they are mixed, ultimately causing nutrient degradation during storage (Petrolii *et al.*, 2020) [22]. Moreover, choline chloride is highly corrosive to metal feed mill equipment, which not only increases maintenance costs but also limits its compatibility with certain premix ingredients (Albers *et al.*, 2002) [1].

In recent years, there has been a growing focus among researchers and the feed industry on natural sources of choline, sourced from plant, algal, and microbial materials. Components found in herbal choline, including phosphatidyl inositol and phosphatidylserine, are essential for the metabolism of phosphatidylcholine, the control of enzymatic processes, and the growth response (Khosravinia *et al.*, 2015) [17]. Research suggests that the incorporation of herbal choline products may alleviate the negative metabolic impacts associated with high-energy diets in broiler chickens. In comparison to synthetic choline, herbal choline does not transform into trimethylamine in the gut, thus avoiding the production of harmful chemicals (Chitithoti *et al.*, 2025) [7]. Rich in choline, they serve as viable substitutes for choline supplementation in diets for broilers. Furthermore, herbal choline possesses reduced hygroscopic properties relative to synthetic choline chloride, ensuring stability and preventing moisture uptake from the air, which aids in maintaining the integrity of the feed (Martinez *et al.*, 2021) [18]. These bioavailable choline complexes—such as those derived from herbal extracts, yeast derivatives, and algae—contain choline in the forms of phosphatidylcholine and glycerophosphocholine, which are absorbed and metabolized with greater efficiency compared to synthetic salts (Perricone *et al.*, 2022; Bhagwat *et al.*, 2024) [21, 5]. Studies show that phosphatidylcholine supplements, like natural choline, might enhance broiler weight gain by improving energy utilisation (efficient metabolism of fat and carbohydrates), without necessarily shifting feed intake or feed conversion ratio (Khosravinia *et al.*, 2015) [17].

In addition to replacing synthetic choline chloride, natural choline substitutes may provide other functional advantages like better liver protection, increased antioxidant capacity, and improved intestinal health. In the production of poultry, it has antibacterial, growth-promoting, immunostimulant, and antioxidative properties. The general performance of birds such as feed intake, body weight gain, feed conversion ratio, nutrient digestibility, carcass characteristics, and egg production, meat quality and safety during storage, and emission reduction in poultry houses are all improved by phytogenic feed additives (Yitbarek, 2015) [27].

Phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine are abundant in plants like *Trachyspermum amni*, *Citrullus colocynthis*, *Achyranthus aspera*, *Azadirachta indica*, *Trigonella foenum-graecum*, *Sida cordifolia*, *Achyranthes aspera*, *Solanum nigrum*,

Ocimum sanctum, *Ocimum basilicum*, *Cichorium intybus*, *Glycine max*, *Curcuma longa*, *Tephrosia purpurea*, *Andrographis paniculata*, etc. These compounds have a higher choline bioavailability than choline chloride, and has hepatoprotective and lipid-lowering effects (Petrolii *et al.*, 2021; Bhagwat *et al.*, 2024) [22, 5], and their combination may produce synergistic benefits. However, their efficacy depends on their concentration, matrix composition, and bioavailability—all of which are still being studied and standardised.

Therefore, the current study was designed to establish a choline-deficient model (CDM) capable of demonstrating variations in body weight gain in broiler chickens fed a basal diet containing either soybean meal (SBM) or maize gluten meal (MGM). Furthermore, this experimentally developed CDM was employed to evaluate and compare the effects of a newly formulated phytogenic herbal formulation called as Natural herbal choline (NHC) and synthetic choline chloride (SCC) on growth performance, serum biochemical parameters, liver histopathology, and carcass characteristics of broiler chickens.

2. Materials and Methods

The present experiment was carried out in the experimental facility of Chauhan poultry farm, Karnal, Haryana, India and all the biochemical and haematological parameters were tested with the help of first choice vet diagnostic center, Safidon, Haryana and New Morden diagnostic lab, Kurukshetra, Haryana.

2.1 Management Practices

The experimental house was cleaned, and disinfected following standard protocol before the arrival of the chicks. All birds were reared in a deep litter system, with uniform floor space, feeder space, and waterer space, and brooding of the chicks was done using incandescent bulbs as per the standard practice. The brooder guard was fixed and arranged from 0-7 days of age. All the chicks were provided with uniform brooding facilities. The lighting regimen practiced was 24 h light: 0 h dark during the first two weeks of age. Adequate care was taken to provide optimum and uniform management to all the treatment groups.

2.2 Procurement of chicks:

A total number of 896 day-old straight-run Ross 308 broiler chicks (*Gallus gallus domesticus*) were procured from Bright Hatchery, Panipath, Haryana, for the study of the experiment. The day-old chicks were individually weighed, and allotted randomly to 3 experimental groups with four replicates in each group.

2.3 Procurement of Natural choline powder (Choliherb):

The feed was formulated as per the BIS standards and procured from the local supplier. Standard feeding practices were followed uniformly. Feed and fresh drinking water were provided ad libitum through semi-automatic feeders & semi-automatic drinkers.

The Choliherb is a proprietary developed by M/s. Regen Biocorps, 301-D/E, 3rd Floor, Ananta Trendz, Gotri-Vuda Road Gotri, Vadodara-390021, Gujrat (INDIA). which contain ingredients of herbs like *Trigonella foenum-graecum*, *Sida cordifolia*, *Achyranthes aspera*, *Solanum nigrum*, *Ocimum sanctum*, *Trachyspermum ammi*, and *Azadirachta indica*. M/s. Regen Biocorps, 301-D/E, 3rd

Floor, Ananta Trendz, Gotri-Vuda Road Gotri, Vadodara-390021, Gujrat (INDIA).

2.4 Feeding Management

Circular feeders and circular drinkers were kept inside the pens to provide sufficient feeding and watering space for the birds. The birds were provided 450 and 929 cm² floor spaces per bird during the brooding and growing periods, respectively. The feeding space provided was 3.0 and 7.0 cm per bird during the brooding and growing period, respectively. The watering space provided per bird was 1.5 and 3.0 cm during the brooding and growing period, respectively. Group feeding practice was followed for all the replicates throughout the experimental period. Weighed amount of feed was offered every day to all the groups and refusal was weighed at the end of each week. By using these data, average weekly feed consumption was calculated for each treatment group and expressed as gm/bird. The birds were given free access to fresh, clean and wholesome water throughout the experimental period.

2.5 Feed Preparation: The feed ingredients used in the present experiment were purchased from local market and rations were prepared as per BIS Indian Standards (2007). The ration was computed as per the regulations of Bureau of Indian Standards (BIS), 2007 as shown in Table I to III.

2.6 Experimental Design

The research consisted of three consecutive experiments. The first experiment aimed to investigate the effect of a choline deficient model (CDM) on the zootechnical parameters of Ross 308 broiler birds. CDM was established in two hundred eighty-eight 1-day-old male Ross-308 broiler chicks, which were randomly divided into four groups with nine replicates, each containing eight birds on the initial day of the experiment. Treatment group 1 (T₁) served as normal controls and was fed basal diet supplemented with SCC, respectively, with 100% soybean meal (SBM) offered as a major protein source. In treatment groups T₂, T₃, and T₄, SBM was replaced as a protein source at 30%, 40%, and 60% by Maize gluten meal (MGM), respectively. The diet of all four treatment groups was formulated to meet the requirements for all nutrients, according to the regulations of NRC (1994), except the choline levels. The replacement of SBM by MGM at various levels was employed to induce a graded level of choline deficiency in chickens (Table IV).

Experiment number two was conducted to investigate the comparative efficacy of a natural polyherbal solution against synthetic choline chloride (SCC) in a choline-deficient model. In this study, three hundred twenty 1-day-old male Ross-308 broiler chicks were randomly assigned to five groups, with each group comprising 8 replicates of 8 birds on the first day of the experiment. Treatment group T₁ served as normal controls and fed basal diet supplemented without SCC. Group T₂ was assigned as CDM control without any SCC, T₃ as CDM with SCC @ 1000g/ton feed, T₄ & T₅ as CDM with low (250 g) and high (500 g) of natural herbal choline (NHC) respectively. The purpose of this experiment was to analyse the efficacy of NHC at two varying dose levels (250 and 500 g/ton feed) compared to SCC, which was administered at a dose of 1000 g/ton feed (Table V).

Experiment number 3 was conducted to investigate the dose-response assessment of NHC in a choline deficient model (CDM) involving broilers. In this study, two hundred eighty-eight 1-day-old male Ross-308 broiler chicks were randomly allocated into six groups, each consisting of 8 replicates with 6 birds per replicate on the initial day of the experiment. The aims of this study were to ascertain the dose-response of NHC, evaluate the consistency of results for NHC at 250 g/ton, and determine if doses lower than 250 g/ton would yield an efficacy comparable to SCC (Table VI).

2.7 Serum biochemistry

2.7.1 Determination of serum aspartate aminotransferase (AST) activity

On day 42, blood samples were collected and centrifuged at 3,000 rpm for 5 minutes, and the serum was collected. According to the protocol outlined in Aspartate Aminotransferase (AST) Test Kit (Span Diagnostic Ltd., Surat, Gujarat, India), 20 µL of the samples and 200 µL of the AST reagent were added to a 96-microwell plate, mixed thoroughly by pipetting, and aspirated for measurement. The absorbance was recorded after 60 seconds, followed by readings taken every 30 seconds until reaching 120 seconds at a wavelength of 340 nm. The average absorbance was calculated per minute, and the AST activity (IU/L) was computed using the following formula: Mean absorbance × 1,768 (kinetic factor).

2.7.2 Determination of serum alanine aminotransferase (ALT) activity

On day 42, blood samples were collected and centrifuged at 3,000 rpm for 5 minutes, and the serum was collected. According to the protocol outlined in Aspartate Aminotransferase (AST) Test Kit (Span Diagnostic Ltd., Surat, Gujarat, India), 20 µL of the samples and 200 µL of the AST reagent were added to a 96-microwell plate, mixed thoroughly by pipetting, and incubated at 37 °C for 1 minute and aspirated for measurement. Absorbance was recorded at intervals of 1, 2, and 3 minutes at a wavelength of 340 nm. The change in absorbance per minute was assessed by taking the mean of the consecutive readings. The AST activity (IU/L) was calculated using the following equation: Mean absorbance × 1,746 (kinetic factor).

2.8 Carcass traits and muscle lipid profile

2.8.1 Determination of cholesterol level

A sample of breast muscle along with abdominal fat, weighing approximately 250 mg, was homogenized in 2,500 µL of 10 mM phosphate-buffered saline (PBS). Subsequently, it was centrifuged at 1,600 rcf for 10 minutes at a temperature of 40 °C. The supernatant was then collected for use in the assay. According to the protocol of Cholesterol Test Kit (Span Diagnostic Ltd., India), 2.5 µL of samples or reagent 2 (standard) was added to separate 96-microwell plates, and 250 µL of the total cholesterol reagent was added, mixed thoroughly by pipetting, and incubated at 37 °C for 10 minutes. The absorbance was read at a wavelength of 505 nm and then cholesterol concentration (mg/dL) was calculated as follows:

$$\frac{\text{Test absorbance}}{\text{standard absorbance}} \times 200.$$

2.8.2 Determination of triglyceride level

A sample of breast muscle along with abdominal fat, weighing approximately 250 mg, was homogenized in 2,500 μ L of 10 mM phosphate-buffered saline (PBS). Subsequently, it was centrifuged at 1,600 rcf for 10 minutes at a temperature of 40 °C. The supernatant was then collected for use in the assay. According to the protocol of Cholesterol Test Kit (Span Diagnostic Ltd., India), 2.5 μ L of samples or reagent 2 (standard) was added to separate 96-microwell plates, and 250 μ L of the total cholesterol reagent was added, mixed thoroughly by pipetting, and incubated at 37 °C for 10 minutes. The absorbance was read at a wavelength of 505 nm and then triglyceride concentration (mg/dL) was calculated as follows:

$$\frac{\text{Test absorbance}}{\text{standard absorbance}} \times 200.$$

3. Results

3.1 Growth performance and parameter

In experiment number 1, replacement of soybean meal with increasing levels of maize gluten meal (MGM) resulted in a marked and dose-dependent deterioration in growth performance of broiler chickens, as represented in Table VII. At 21 days of age, body weight was significantly highest ($P < 0.001$) in birds fed the control diet of 988 g containing 0% MGM (T_1), while progressive reductions were observed with increasing MGM inclusion, reaching the lowest body weight of 654 g in the 60% MGM group (T_4). A similar trend persisted at 42 days, where birds in the T_1 group achieved the highest final body weight of 2820 g, whereas birds receiving 60% MGM (T_4) exhibited significantly lower body weight of 1985 g. Body weight gain followed a comparable pattern. During the starter phase (1-21 days), birds fed the control diet (T_1) recorded the greatest weight gain of 945 g, while weight gain declined significantly ($P < 0.001$) with increasing MGM inclusion, with the lowest gain of 611 g observed in the 60% MGM group (T_4). Over the entire experimental period (1-42 days), cumulative body weight gain was highest in the control group with 2777 g and progressively decreased across the T_2 , T_3 and T_4 groups, with the lowest BW gain recorded in the T_4 group, with weight gain of 1942 g. Feed intake was significantly influenced by dietary MGM level. Birds fed the control diet (T_1) consumed the highest amount of feed (1330 g and 4395 g) during both the starter and overall rearing period, respectively. Feed intake declined significantly ($P < 0.001$) as CGM inclusion increased, with the lowest consumption recorded in the T_4 group during both phases. Feed conversion ratio (FCR) deteriorated significantly with increasing MGM inclusion. During the starter phase, FCR increased from 1.41 in the T_1 group to 1.73 in the T_4 group ($P < 0.001$). Similarly, over the entire rearing period, T_1 group exhibited the most efficient FCR with 1.58, while the poorest feed efficiency was observed in T_4 group @ 1.93. Mortality rates showed a numerical increase with higher CGM inclusion, ranging from 1.4% in T_1 group to 4.2% in T_4 group; however, these differences were not statistically significant ($P > 0.05$). The 40% MGM diet (T_3) was selected for subsequent experiments as it provided a robust and consistent deficiency.

In experiment number 2, birds maintained on the choline-deficient model (CDM) diet exhibited significant depression in growth performance compared with the normal control group, as represented in Table VIII. At 21 days, body

weight of 770 g (T_2 group) was significantly lower ($P < 0.001$) as compared to T_1 . Supplementation with SCC (1000 g/ton) or NHC significantly improved body weight compared with the CDM control group (T_2), with the highest body weight recorded in T_5 group, which was statistically comparable to the normal control. At 42 days, a similar pattern was observed. Birds in the T_2 group recorded the lowest final body weight of 2360 g, whereas supplementation with SCC and NHC resulted in significant improvements. The T_5 group achieved a final body weight of 2775 g compared to body weight of 2815 g represented in T_1 group and superior to SCC supplementation. Body weight gain during both starter and overall phases reflected these trends. Birds fed the CDM diet showed significantly reduced weight gain, whereas supplementation with SCC or NHC significantly restored growth performance. The highest cumulative weight gain at 1-42 days was observed in T_5 group with weight gain of 2732 g as compared to T_1 with weight gain of 2772 g. Feed intake was numerically lower in T_2 group during both phases, however, the differences among treatments were not statistically significant ($P > 0.05$). Despite similar feed intake, FCR was significantly impaired in T_2 group. Supplementation with SCC and NHC significantly improved FCR during both phases, with the most efficient FCR values observed in the T_1 and T_5 groups, respectively. Mortality rates remained low and did not differ significantly among treatments ($P > 0.05$). Both NHC doses showed remarkable efficacy. NHC at 500 g/ton (T_5) significantly outperformed SCC in both BWG and FCR at 42 days, while NHC at 250 g/ton (T_4) showed performance similar to T_3 group.

The dose-response study in experiment number 3 further confirmed the impact of graded NHC supplementation in comparison with SCC (Table IX). At 21 days, body weight was significantly lowest in the T_2 group with BW of 768 grams, while birds supplemented with SCC @ 450 g/ton (T_3) and increasing doses of NHC showed significant improvements ($P < 0.001$). The highest body weight at 21 days was observed in T_6 group with BW of 925 g compared to T_1 with BW of 990 g. At 42 days, final body weight followed a similar pattern. Birds in the T_2 group recorded the lowest body weight of 2355 g, whereas supplementation with SCC and NHC significantly enhanced growth. The T_6 group achieved a final body weight of 2755 g compared to T_1 with BW of 2830 g, representing data superior to lower NHC doses (T_3 , T_4 and T_5 respectively). Body weight gain during both the starter and overall periods improved significantly with increasing NHC inclusion. The highest cumulative weight gain was observed in T_6 group with 2712 g weight gain, which was statistically comparable to the data of T_1 and superior to SCC supplementation (T_3). Feed intake was numerically reduced in the T_2 group but did not differ significantly among treatments ($P > 0.05$). However, FCR was significantly affected. Birds fed the CDM diet exhibited the poorest FCR, while supplementation with SCC and NHC significantly improved feed efficiency. The best overall FCR values were observed in the T_1 and T_6 groups, respectively. Mortality remained low across treatments and was not significantly influenced by dietary treatments.

3.2 Serum biochemistry

Serum liver enzyme activities at 42 days are presented in Table X. Birds fed the CDM diet (T_2) exhibited markedly elevated serum AST (356 U/L) and ALT (38.6 U/L) levels

compared with the normal control group (T_1) with AST and ALT values of 212 and 14.2 U/L, respectively, indicating significant hepatic stress ($P < 0.001$). Supplementation with SCC (T_3) significantly reduced AST and ALT levels compared with the CDM control (T_2). Graded supplementation of NHC resulted in dose-dependent reductions in liver enzyme activities. The T_6 group recorded AST level of 225 U/L and ALT level of 15.8 U/L, compared to T_1 , indicating effective restoration of hepatic function.

3.3 Carcass traits and muscle lipid profile

Breast muscle lipid analysis revealed significant effects of dietary treatments on lipid deposition, as represented in Table XI. Birds fed the CDM diet (T_2) exhibited significantly higher triglyceride of 8.5 mg/g and cholesterol of 0.98 mg/g concentrations as compared with the normal control (T_1) with triglycerides and cholesterol of 4.6 and 0.58 mg/g, respectively ($P < 0.001$). Supplementation with SCC (T_3) significantly reduced lipid accumulation in breast muscle. Graded NHC supplementation resulted in dose-dependent reductions in both triglyceride and cholesterol levels, with the T_6 group showing values of 4.8 mg/g triglycerides and 0.59 mg/g cholesterol, which was almost similar to the T_1 group. Carcass yield was modestly but significantly affected by dietary treatment ($P < 0.05$). Birds fed the CDM diet (T_2) exhibited the lowest carcass yield of 71.4%, whereas the highest yield was observed in the T_6 group with 72.5%, which was similar to the T_1 group.

3.4 Histopathology

The liver of control group shows very normal histoarchitecture (Figure 1). The histopathology revealed mild fatty changes, degenerative changes in hepatic parenchyma and dilatation of central vein in the liver of CDM control group birds (Figure 2). Whereas the group of birds with CDM with SCC @ 450 g/ton (Figure 3) and NHC @ 100 g/ton (Figure 4) show mild congestion and

degenerative changes in hepatic parenchyma. In contrast, reduced swelling, and degenerative changes and almost normal histoarchitecture of liver parenchyma was seen in the group of CDM birds supplemented with 250 g/ton of NHC (Figure 5).

Table 1: Experiment ingredients and chemical composition of Pre-Starter feed

S. No.	Ingredients	T_1	T_2	T_3	T_4
1	Maize	532	520	520	520
2	Soyabean meal	335	251	201	201
3	Soya protein isolate	39	16	4	4
4	Maize gluten meal 42	0	84	134	134
5	De oiled rice bran	16	51	61	61
7	Vegetable Oil	37.5	37.5	37.5	37.5
8	Lime stone powder (LSP)	10	12	14	14
9	Dicalcium Phosphate (DCP)	20	16	14	14
10	Sodium Chloride	2	2	2	2
11	Trace mineral Premix	1	1	1	1
12	Vitamin Premix	0.5	0.5	0.5	0.5
13	L-Lysine	1.5	4.5	6.5	6.5
14	Synthetic choline chloride Powder (60%)	1	0	0	0
15	Coccidiostat	0.5	0.5	0.5	0.5
16	Sodium bicarbonate	1	1	1	1
17	Toxin binder	1	1	1	1
18	DL-Methionine	2	2	2	2
Nutrient composition (Calculated value)					
1	Metabolizable Energy (Kcal/Kg)	3002.2	3004.4	3020.2	3023.8
2	Crude Protein (%)	23.05	23.04	23.08	23.02
3	Lysine (%)	1.47	1.46	1.49	1.48
4	Methionine (%)	0.56	0.56	0.57	0.57
5	Calcium (%)	0.99	0.96	0.98	0.97
6	Available Phosphorus (%)	0.49	0.49	0.49	0.5
7	Crude Fibre (%)	3.55	3.73	3.66	3.87
8	Ether Extract (%)	6.14	6.41	6.57	6.59
9	Choline (PPM)	1691.1	1037.8	911.04	846.97

Table 2: Experiment ingredients and chemical composition of Starter feed

S. No.	Ingredients	T_1	T_2	T_3	T_4
1	Maize	579	590	594	600
2	Soyabean meal	303	227	182	121
3	Soya protein isolate	35	39	27	44
4	Maize gluten meal 42	0	42	85.5	99
5	De oiled rice bran	3	25	38	63.5
7	Vegetable Oil	43	39	36	34
8	Lime stone powder (LSP)	9	10	12	14
9	Dicalcium Phosphate (DCP)	18	17	14	12
10	Sodium Chloride	2	2	2	2
11	Trace mineral Premix	1	1	1	1
12	Vitamin Premix	0.5	0.5	0.5	0.5
13	L-Lysine	1	2.5	3.5	4.5
14	Synthetic choline chloride Powder (60%)	1	0	0	0
15	Coccidiostat	0.5	0.5	0.5	0.5
16	Sodium bicarbonate	1	1	1	1
17	Toxin binder	1	1	1	1
18	DL-Methionine	2	2.5	2	2
Nutrient composition (Calculated value)					
1	Metabolizable Energy (Kcal/Kg)	3101.1	3108.7	3106.2	3110.6
2	Crude Protein (%)	21.54	21.56	21.5	21.52
3	Lysine (%)	1.31	1.29	1.26	1.33
4	Methionine (%)	0.53	0.54	0.54	0.55
5	Calcium (%)	0.88	0.88	0.88	0.9
6	Available Phosphorus (%)	0.43	0.44	0.44	0.44
7	Crude Fibre (%)	3.2	3.1	3.2	3.3
8	Ether Extract (%)	6.83	6.45	6.49	6.36
9	Choline (PPM)	1609.7	1012.6	901.7	806.5

Table 3: Experiment ingredients and chemical composition of Finisher feed

S. No.	Ingredients	G1	G2	G3	G4
1	Maize	600	608.5	616	617
2	Soyabean meal	277	208	167	111
3	Soya protein isolate	32	29	41.5	50
4	Maize gluten meal 42	0	45	55	80
5	Deoiled rice bran	0	21	21	44
6	Natural choline (NCF)	1	1	13	13
7	Vegetable Oil	55	51	50	48
8	Lime stone powder (LSP)	8	8.5	8.5	9
9	DCP	17	18	18	18
10	NaCl	2	2	2	2
11	Trace mineral Premix	1	1	1	1
12	Vitamin Premix	0.5	0.5	0.5	0.5
13	L-Lysine	1.5	2.5	2.5	3.25
14	Synthetic choline chloride Powder (60%)	1	0	0	0
15	Coccidiostat	0.5	0.5	0.5	0.5
16	Sodium bicarbonate	0.5	0.5	0.5	0.5
17	Toxin binder	1	1	1	1
18	DL-Methionine	2	2	2	1.75
Nutrient composition (Calculated value)					
1	ME (Kcal/Kg)	3202.5	3200.4	3200.6	3201.8
2	CP (%)	20.29	20.18	20.12	20.27
3	Lysine (%)	1.27	1.21	1.19	1.2
4	Methionine (%)	0.51	0.52	0.52	0.5
5	Calcium (%)	0.81	0.84	0.83	0.84
6	Available Phosphorus (%)	0.405	0.41	0.41	0.42
7	Crude Fibre (%)	3.01	3.03	2.8	2.9
8	Ether Extract (%)	8.08	7.87	7.82	7.71
9	Choline (PPM)	1543	949.7	872.8	773.8

Table 4: Effect of choline deficient model (CDM) in zootechnical parameters in Ross 308 broiler birds

Treatment	T ₁ (Normal control)	T ₂ (30% MGM)	T ₃ (40% MGM)	T ₄ (60% MGM)
Number of birds	72	72	72	72
Soyabean Meal (%)	100	70	60	40
Maize gluten meal (%)	0	30	40	60
SCC (g/ton)	1000	0	0	0

Table 5: Evaluation of efficacy of NHC at two different dose levels (250 and 500 g/ton feed)

Treatment	T ₁ (Normal control)	T ₂ (CDM Control)	T ₃ (CDM + SCC)	T ₄ (CDM + NHC-L)	T ₅ (CDM + NHC-H)
Number of birds	64	64	64	64	64
Soyabean Meal (%)	100	0	60	60	60
Maize gluten meal (%)	0	40	40	40	40
SCC (g/ton)		0	1000	0	0
Natural herbal choline (g)				250 g	500 g

Table 6: Dose-response evaluation of NHC in choline deficient model (CDM)

Treatment	T ₁ (Normal control)	T ₂ (CDM Control)	T ₃ (CDM + SCC)	T ₅ (CDM + NHC-100)	T ₄ (CDM + NHC-200)	T ₅ (CDM + NHC-250)
Number of birds	48	48	48	48	48	48
Soyabean Meal (%)	100		60	60	60	
Maize gluten meal (%)	0	40	40	40	40	
SCC (g/ton)		0	450	0	0	
Natural herbal choline (g)				100	200	250

Table 7: Effect of choline deficient meal (CDM) on Zootechnical Parameters in Ross 308 broiler chicken.

Treatment	Day	T ₁ (Normal Control)	T ₂	T ₃	T ₄	Pooled SEM	p-value
Body Weight (g)	21	988 ^a	875 ^b	765 ^c	654 ^d	14.8	<0.001
	42	2820 ^a	2545 ^b	2355 ^c	1985 ^d	27.5	<0.001
Body Weight Gain (g)	1-21	945 ^a	832 ^b	722 ^c	611 ^d	14.7	<0.001
	1-42	2777 ^a	2502 ^b	2312 ^c	1942 ^d	27.4	<0.001
Feed Intake (g)	1-21	1330 ^a	1270 ^b	1185 ^c	1055 ^d	17.2	<0.001
	1-42	4395 ^a	4260 ^b	4125 ^c	3750 ^d	30.8	<0.001
FCR	1-21	1.41 ^a	1.53 ^b	1.64 ^c	1.73 ^d	0.02	<0.001
	1-42	1.58 ^a	1.70 ^b	1.78 ^c	1.93 ^d	0.02	<0.001
Mortality (%)	1-42	1.4	2.1	2.8	4.2	0.6	0.121

The groups with different superscript in a row differ significantly (P<0.01)

Table 8: Comparative evaluation of SCC and NHC in choline deficient model

Treatment	Day	T ₁ (Normal Control)	T ₂ (CDM Control)	T ₃	T ₄	T ₅	Pooled SEM	p-value
Body Weight (g)	21	985 ^a	770 ^d	900 ^b	910 ^b	945 ^a	11.8	<0.001
	42	2815 ^a	2360 ^d	2660 ^b	2695 ^b	2775 ^a	22.5	<0.001
Body Weight Gain (g)	1-21	942 ^a	727 ^d	857 ^b	867 ^b	902 ^a	11.7	<0.001
	1-42	2772 ^a	2317 ^d	2617 ^b	2652 ^b	2732 ^a	22.4	<0.001
Feed Intake (g)	1-21	1325	1190	1260	1275	1305	14.9	0.067
	1-42	4380	4130	4285	4310	4355	28.1	0.089
FCR	1-21	1.41 ^a	1.64 ^d	1.47 ^b	1.47 ^b	1.45 ^{ab}	0.02	<0.001
	1-42	1.58 ^a	1.78 ^d	1.64 ^b	1.63 ^b	1.59 ^a	0.01	<0.001
Mortality (%)	1-42	1.3	2.5	1.9	1.3	0.6	0.5	0.301

The groups with different superscript in a row differ significantly (P<0.01)

Table 9: Dose response evaluation of NHC in CDM model in comparison with SCC

Parameter	Day	T ₁ Normal Control	T ₂ CDM Control	T ₃	T ₄	T ₅	T ₆	Pooled SEM	p-value
Body Weight (g)	21	990 ^a	768 ^d	885 ^b	795 ^c	855 ^{bc}	925 ^a	12.5	<0.001
	42	2830 ^a	2355 ^d	2675 ^b	2450 ^c	2585 ^c	2755 ^a	24.8	<0.001
Body Weight Gain (g)	1-21	947 ^a	725 ^d	842 ^b	752 ^c	812 ^{bc}	882 ^a	12.4	<0.001
	1-42	2787 ^a	2312 ^d	2632 ^b	2407 ^c	2542 ^c	2712 ^a	24.7	<0.001
Feed Intake (g)	1-21	1335	1185	1265	1210	1250	1295	15.5	0.071
	1-42	4400	4120	4290	4205	4275	4340	29.8	0.101
FCR	1-21	1.41 ^a	1.63 ^d	1.50 ^b	1.61 ^c	1.54 ^{bc}	1.47 ^{ab}	0.02	<0.001
	1-42	1.58 ^a	1.78 ^d	1.63 ^b	1.75 ^c	1.68 ^{bc}	1.60 ^a	0.01	<0.001
Mortality (%)	1-42	1.6	2.1	1.6	2.1	1.6	0.0	0.5	0.785

The groups with different superscript in a row differ significantly (P<0.01)

Table 10: Serum Biochemistry of the treatment groups

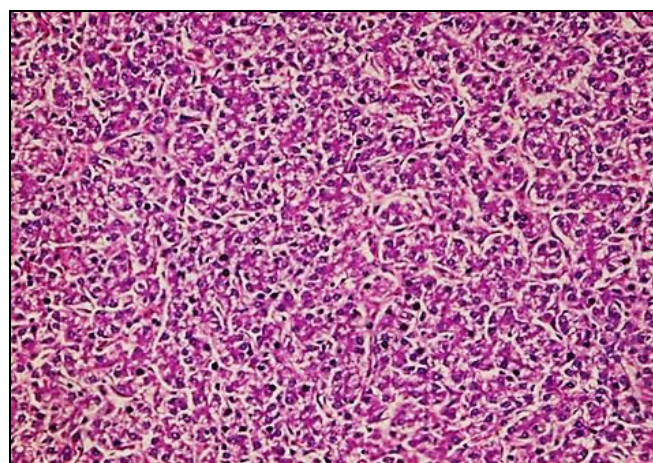
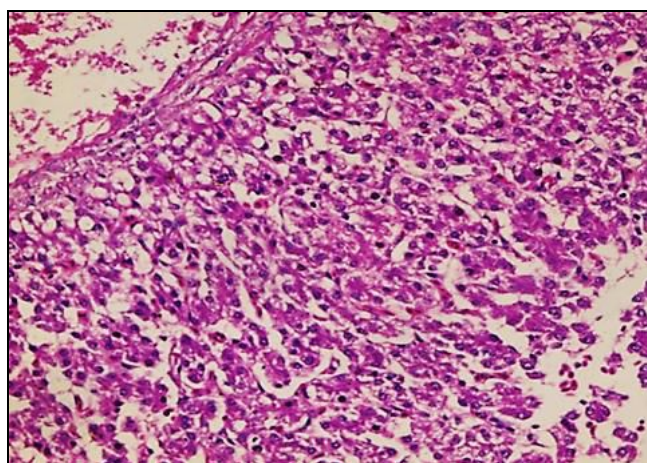
Serum Biochemistry	Day	T ₁ Normal Control	T ₂ CDM Control	T ₃	T ₄	T ₅	T ₆	Pooled SEM	p-value
AST (U/L)	42	212 ^a	356 ^c	248 ^b	310 ^d	270 ^c	225 ^a	8.4	<0.001
ALT (U/L)	42	14.2 ^a	38.6 ^c	18.9 ^b	32.4 ^d	24.6 ^c	15.8 ^a	1.6	<0.001

Means in a row with different superscripts are significantly different (p<0.05).

Table 11: Carcass Traits and Muscle lipid profile

Breast Muscle Lipids	Day	T ₁ Normal Control	T ₂ CDM Control	CT ₃ DM + SCC (450 g/t)	T ₄ CDM + NHC-100	T ₅ CDM + NHC-200	T ₆ CDM + NHC-250	Pooled SEM	p-value
Triglycerides (mg/g)	42	4.6 ^a	8.5 ^d	5.3 ^b	7.2 ^c	6.1 ^b	4.8 ^a	0.3	<0.001
Cholesterol (mg/g)	42	0.58 ^a	0.98 ^d	0.65 ^b	0.85 ^c	0.72 ^b	0.59 ^a	0.03	<0.001
Carcass Yield (%)	42	72.3 ^a	71.4 ^b	71.8 ^{ab}	71.6 ^b	71.9 ^{ab}	72.5 ^a	0.4	0.045

Means in a row with different superscripts are significantly different (p<0.05).

**Fig 1:** Control group: Normal Histoarchitecture of liver parenchyma (H & E, 400X)**Fig 2:** CDM control group: mild fatty infiltration and degenerative changes in hepatic parenchyma and dilatation of central vein (H & E, 400X).

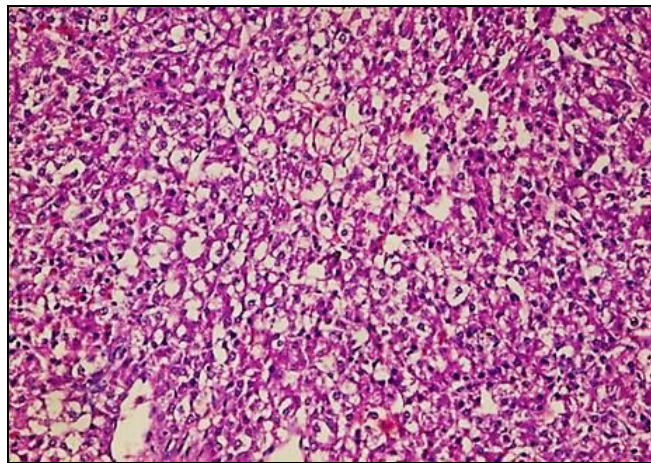


Fig 3: CDM + SCC 450 g/ton: Section of liver showing mild fatty changes and degenerative changes in hepatic parenchyma (H & E, 400X).

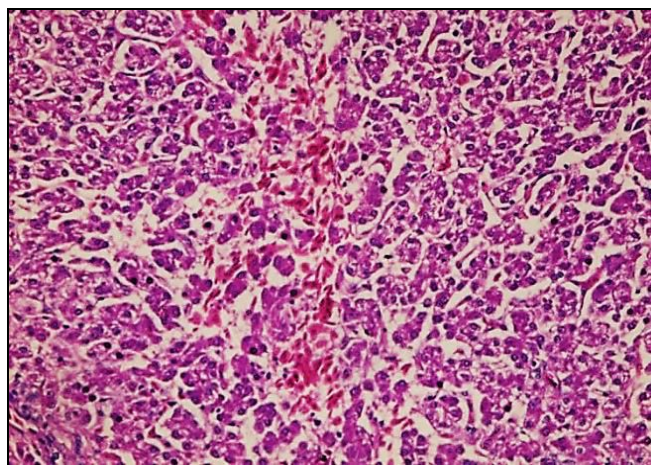


Fig 4: CDM + 100 g/ton NHC: mild congestion and degenerative changes in hepatic parenchyma (H & E, 400X).

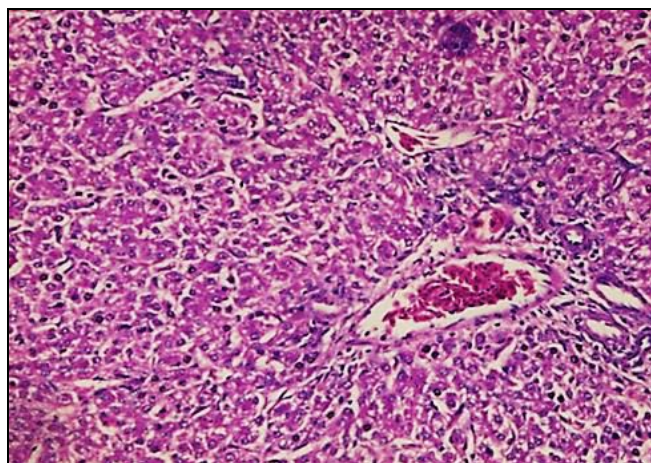


Fig 5: CDM + 250 g/ton NHC: Without appreciable histopathological change and almost normal histoarchitecture of liver parenchyma. (H & E, 400X).

4. Discussion

Choline is a vital nutrient in poultry nutrition, significantly contributing to the prevention of metabolic disorders like fatty liver syndrome, perosis, and reduced growth performance in broiler chickens. While dietary methionine can serve as a methyl group donor and, in theory, aid in the endogenous synthesis of choline, young chicks possess a naturally limited ability to synthesize choline. This

limitation is primarily due to the ineffectiveness of S-adenosyl-methionine as a methyl donor in the initial methylation phase of phosphatidylethanolamine, a crucial step in the biosynthesis of choline. (Jukes, *et al.* 1945; Baker & Sugahara, 1970) [14, 4]. Consequently, reliance on endogenous synthesis alone is insufficient to meet the physiological demand for choline during early growth stages.

Furthermore, a substantial portion of dietary choline undergoes irreversible oxidation to betaine, which restricts its reutilization as a precursor for choline synthesis. While both methionine and betaine can partially substitute for choline by contributing methyl groups to the one-carbon metabolic pool, this substitution addresses only one aspect of choline's multifunctional role. Choline is additionally required for the synthesis of acetylcholine and phospholipids, which are vital for neurotransmission, cellular membrane integrity, and lipid transport (Arele *et al.*, 2015; Wauben and Wainwright, 1999) [2, 25]. These functions appear to be more critical than its role as a methyl donor, as evidenced by earlier studies demonstrating that betaine supplementation fails to improve growth performance in birds fed a choline-free purified diet unless a substantial proportion of the choline requirement is met through direct choline supplementation (Gregg *et al.*, 2023) [12].

According to the Ross-308 manual, Aviagen 2022-2025, the dietary choline requirements of broiler chickens to support optimal body weight gain and feed consumption are estimated to be approximately 1700 mg/kg during the starter phase, 1600 mg/kg during the grower phase and 1500 mg/kg during the finisher phase. Earlier investigations have established choline requirements and assessed choline availability in different feed ingredients using purified diets based on corn starch and isolated soybean protein (Dilger *et al.*, 2007; Emmert & Baker, 1997, Molitoris & Baker, 1976) [8, 9, 19]. Despite this, limited research has specifically addressed the physiological and production-related consequences of choline deficiency in broilers, including impaired growth performance, deterioration in feed conversion efficiency, and the development of fatty liver.

Additionally, several studies examining the role of supplemental synthetic choline chloride in practical poultry diets have reported inconsistent responses, largely because conventional corn-soybean meal-based diets generally provide sufficient choline to meet the birds' nutritional requirements, irrespective of supplemental choline inclusion (NRC, 1994) [25]. This inherent adequacy of choline in standard commercial diets makes it difficult to observe a measurable biological response to choline supplementation. Therefore, the development of an appropriate experimental model capable of inducing a measurable choline deficiency is essential to accurately evaluate the effects of dietary choline supplementation. In this context, soybean meal was partially or completely replaced with maize gluten meal and soya protein isolate as the primary protein source in the diet to reduce endogenous choline contribution, and the resulting effects on growth performance and key zootechnical parameters were assessed in the first experiment.

Replacing SBM with MGM induced classical choline deficiency symptoms, such as reduced growth, higher FCR, and disturbed lipid metabolism. The replacement of SBM with MGM and Soya protein isolate causes reduced choline content in the formulated diet as the choline content of maize gluten meal (0.363 g/kg) was very less as compare to

soyabean meal (2.64 g/Kg). The dose-dependent impairment in BWG and FCR with decreasing SBM and increasing MGM inclusion (25%, 40% and 60%) confirms the induction of choline deficiency. This observation was in accordance with the reports by Farina *et al.*, 2017, Dilger, 2007 and, Emmert & Baker, 1997^[10, 8, 9] who found an almost linear response to the incremental supplementation of choline chloride in chicks fed choline-deficient basal diet, indicating the positive role of supplemental choline on weight gain of birds. Besides, perosis and growth depression have been reported as the characteristic symptoms of choline deficiency in ducks (Wen *et al.*, 2014)^[26]. It also agrees with previous findings that plant-based protein sources vary in choline content (Avi *et al.*, 2025)^[3] and that MGM-based diets can effectively induce deficiency (Giannenas *et al.*, 2017)^[11]. The 40% MGM inclusion level produced consistent effects without high mortality confirming it as an effective CDM, aligning with earlier CDM designs (Emmert & Baker, 1997)^[9].

Across both Experiments 2 and 3, NHC consistently matched or exceeded SCC in improving BW, BWG, and FCR. This supports recent research showing that herbal sources of biologically active methyl donors (e.g., phosphatidylcholine-rich extracts, hepatoprotective botanicals) can be absorbed more efficiently than crystalline choline chloride (Chitithoti *et al.*, 2025)^[7]. Herbal choline analogues may exhibit slower release kinetics, improved membrane incorporation, and synergistic hepatoprotective phytochemicals which together enhance bio efficacy compared with SCC (Kannan *et al.*, 2013; Selvam *et al.*, 2018)^[15, 24].

These outcomes agree with recent findings that phytogenic choline sources can effectively replace synthetic choline chloride due to better stability, bioavailability, and hepatoprotective phytochemicals (Khose *et al.*, 2018)^[16]. The superior performance of NHC has also been linked to antioxidant capacity and improved methyl group metabolism (Savaram *et al.*, 2021)^[23].

Therefore, diets that are severely deficient in choline are unlikely to show a positive growth response to supplementation with methionine or betaine alone. In contrast, diets with marginal choline insufficiency may respond favourably to these methyl donors due to partial compensation of methyl group availability. Collectively, these observations emphasize the indispensable role of choline itself in poultry diets and underscore the necessity of ensuring adequate dietary choline supply to support optimal growth, liver function, and overall metabolic health in broiler chickens.

Dose-Response and Practical Relevance of the study

NHC exhibited a clear dose-response curve, with **250 g/t** repeatedly matching or exceeding SCC performance. This is especially valuable for commercial poultry production because:

- SCC prices fluctuate widely
- SCC loses potency due to hygroscopic degradation
- NHC provides multifunctional benefits (hepatoprotection + methyl donation) lower inclusion rates reduce diet cost
- These findings align with industry observations that phytogenic supplements can reduce reliance on synthetic additives (Brenes & Roura, 2020)^[6].

Conclusion

This study indicates that the incorporation of a phytogenic choline formulation markedly improved growth performance and feed utilization efficiency, enhanced lipid profiles and carcass traits, and restored liver function in broilers, whereas inadequate levels of dietary choline negatively impacted the growth performance of broiler chickens. A dependable model of choline deficiency was created by designing diets that substitute 40% of soybean meal with maize gluten meal and soya protein isolate, which successfully elicited the typical growth-related manifestations of choline deficiency. This model has been validated as an effective tool for evaluating feed additives that exhibit choline-like properties. In addition, it showed that NHC supplementation at 250 g/ton could successfully substitute for 1 kg/ton of synthetic choline chloride (60%), as indicated by improved growth metrics and enhanced feed efficiency.

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