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Research Article
Effects of a Plant Based Natural Alternative of Choline and Betaine as Replacements for Synthetic Choline in Broilers Diets

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Abstract
Objective: This study aimed to assess the feasibility of replacing synthetic Choline Chloride (CC) with a natural Choline Alternative (NC) and betaine (BT) in broiler diets, focusing on their effects on broiler performance. **Materials and Methods:** Ten dietary treatments were tested on 3,000 male Ross-308 broiler chicks over a 42-day period, including varying levels of Natural Choline (NC), betaine (BT) and synthetic Choline Chloride (CC), with 10 replicates per treatment. Birds were housed in 100 pens, with each pen serving as an experimental unit. **Results:** The study found a significant effect of diet on BW at 42 days. Broilers fed 400 g/ton of either BT or NC exhibited higher BW compared to the control, birds fed with 200 g/ton of NC and broilers fed with CC. The most efficient Feed Conversion Ratio (FCR) was observed in broilers fed the combination of NC 100 g/ton and BT 200 g/ton, followed by those fed BT or NC at 200 g/ton each. No significant differences ($p>0.05$) were observed among these treatments. Broilers supplemented with CC and the control group showed the least efficient FCR. The combination of NC and BT showed better performance. **Conclusion:** These findings suggest that NC and BT can replace synthetic CC in broiler diets without compromising performance. Moreover, the combination of NC and BT may provide a synergistic benefit, potentially leading to better feed efficiency and growth performance. This indicates that natural choline alternatives can be a viable replacement for synthetic choline chloride in broiler nutrition.

Key words: Betaine, broiler, choline chloride, growth performance, natural choline

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Choline, a rediscovered B4 vitamin, is mainly present in the form of phospholipids and plays a crucial role in various biological functions in poultry[1]. It is involved in lipid transport, cell signaling and the biosynthesis of methylated compounds. In poultry, choline promotes digestion and the use of feed energy. It is a component of membrane phospholipids that participate in hepatic lipid metabolism and prevents fat accumulation in the liver, as demonstrated in chicken models[2-4]. Choline also serves as a precursor to acetylcholine, a neurotransmitter responsible for nerve synapses[5]. In response to the dynamic demands of the market, genetic selection has driven the development of highly muscular and efficient broiler chickens. Presently, choline deficiency is not commonly observed in commercial broiler chickens. However, with ongoing genetic advancements, the nutritional needs of modern broilers are likely to undergo changes[6]. The current dietary recommendations for choline in broiler diets, based on the NRC[7], are 1300 mg kg^{-1} of feed, assuming that sufficient levels of sulfur amino acids are also present.

Choline is essential for various biological functions due to its active methyl groups, converting homocysteine to methionine, after being oxidized to betaine (BT) and acting as a lipotropic factor to prevent excessive lipid accumulation and fatty liver[8,9]. While the body can synthesize choline *de-novo*, inadequate production and/or supply may lead to deficiency symptoms in young chicks, such as growth retardation and perosis. The bioavailability of dietary choline varies based on several factors, including other methyl donors like dietary methionine which cannot fully replace the need for choline[7]. BT as a methyl donor provides methyl groups, which are essential for animal metabolism. By adding BT to the diet, the body is enriched with methionine, which promotes protein synthesis[10,11].

Adding synthetic Choline Chloride (CC) to broiler chicken feed has become necessary to meet choline requirements[12,13]. However, CC has several disadvantages, such as a high hygroscopicity, vitamin losses due to oxidation and the formation of trimethylamine by the gut microbiota, prompting researchers to explore alternatives to synthetic CC in animal feeds[14]. Plant-based preparations and compounds have been studied as potential substitutes for CC. A Natural Choline (NC) composed of *Solanum nigrum*, *Trachyspermum ammi* and *Achyranthes aspera* shows potential for enhancing growth performance and replace synthetic CC[15,16]. This NC offers superior bioavailability due to its content of phosphatidylcholine and other essential metabolites. Additionally, its effectiveness is not solely attributed to phospholipids, but also to a diverse range of bioactive compounds like saponins, flavonoids and thymol, which contribute significantly to the efficient recycling of amino acids, vitamins and fatty acids[15,17,18].

Therefore, this study aimed to assess the effectiveness of NC and BT as substitutes for synthetic CC in broiler chicken diets for a duration of 42 days. The primary focus was to validate the potential of BT and NC in replacing CC, while also examining the synergistic effects between BT and NC due to their distinct mechanisms of action.

MATERIALS AND METHODS

Animal care and use: The study was conducted by authorized, qualified and trained veterinarians, scientists and technicians in compliance with the principles of EU Directive 2010/63/EU regarding the protection of animals used for experimental and other specific purposes.

Animal husbandry: The trial was carried out with the research services of ZOOTESTS, Ploufragan, France and within a commercial barn (SCEA CA Elevage Pluzerec, 22320, Plussulien, Brittany, France). The broiler house used for this experiment was a Colorado type with dynamic transverse ventilation which measured 1200 m^2. On one side of the barn, 100 testing pens (Length$\times$Width$\times$Height = 1.90$\times$1.25$\times$0.80 m; 30 birds/pen) were set and separated by metal grids. Pens were covered with wood shavings and were equipped with 3 nipple drinkers in the first 1/3 of the pen and with a 15 kg capacity feeder in the second 1/3 of each pen filled manually. The lighting program was: 24 h a day of lighting with 100% light intensity, from 0-4 days; from day 5-41, a total of 18 h a day of lighting (16 D – 4 N – 2 D – 2 N) with 30-40 % light intensity. Wood shavings were used as litter material (8 kg/pen at the starting time) until 42 days and pen litter scores were assessed by following ITAVI protocol. Body Weight (BW), Feed Intake (FI) and litter quality were measured at D13, 21, 28, 35 and 42. Mortality and temperature were recorded daily. Room temperature was maintained at 33$\pm$1°C during the 1st week; thereafter, the temperature was routinely lowered to 24$\pm$1°C, while the humidity was kept at 60%. Average FI and Feed Conversion Ratio (FCR) were corrected for mortality. Day-old chicks were sexed and vaccinated against infectious bronchitis with H120 strain (aerosol spraying) and at 17 days of age the birds were vaccinated with Gumboro vaccine (G97 strain) via the drinking water.

Dietary treatments: A total of 3,000 day-old male chicks (ROSS 308) were weighed and randomly distributed into the 10 treatments with 10 replicate pens each (n = 10), containing 30 chicks/pen (Table 1). The treatments were designed to

Table 1: Description of dietary treatments

Treatments	n	Natu-B4™ (NC) (g/ton)	Betaine (BT) (g/ton)	Choline chloride (CC) 60% (g/ton)
T1	10	0	0	0
T2	10	100	0	0
T3	10	200	0	0
T4	10	400	0	0
T5	10	0	200	0
T6	10	0	400	0
T7	10	0	0	480
T8	10	0	0	960
T9	10	100	200	0
T10	10	100	0	480

Table 2: Composition of male broiler diets (%, as fed basis) for 42 days

	Starter (day 0-13)	Grower (day 14-28)	Finisher (day 29-42)
Ingredient (%)			
Corn	35.7	22.9	17.7
Wheat	25	40	50
Soybean meal	26.7	21.4	16
Rape seed meal	3	5	5
Soy hulls	3.3	3	3
Soybean oil	1	1	1
Premix	0.5	0.5	0.5
Lime, fine	1.45	1.26	1.23
MCP	1.65	1.4	1.2
Palm fat		2	2.8
Salt	0.15	0.15	0.15
$NaHCO_3$	0.35	0.35	0.35
L-lysine	0.385	0.365	0.38
Dl-methionine	0.33	0.29	0.255
L-threonine	0.185	0.165	0.165
L-valine	0.095	0.085	0.09
L-isoleucine	0.07	0.075	0.085
L-arginine	0.11	0.105	0.135
Total	100	100	100
Calculated values			
Crude protein (%)	20.7	19.4	17.6
Calcium (%)	0.9	0.8	0.75
Available phosphorus (%)	0.48	0.48	0.38
Dig. Lysine (%)	1.15	1.042	0.929
Dig. Methionine (%)	0.586	0.529	0.470
Metabolizable energy, kcal/kg	2800	2900	3000
Crude fat (%)	3.65	5.32	5.97
Crude fiber (%)	3.85	3.87	3.80
Crude ash (%)	6.2	5.64	5.16
Choline, g/ton (calc.)	1348	1420	1350

MCP, Mono dicalcium phosphate

meet both scientific and commercial objectives. The treatments included a negative control (T1) to assess natural broiler performance without any additional choline source. The NC was tested at 100, 200 and 400 g/ton in Treatments 2 (T2), 3 (T3) and 4 (T4) to determine its effects at different doses. The BT at 200 and 400 g/ton were used respectively in treatments 5 and 6 (T5) to compare BT's effectiveness without NC and CC. Synthetic CC was used as the choline source in treatments 7 (T7) and 8 (T8) at 480 and 960 g/ton, respectively. In Treatments 9 (T9) and 10 (T10), low levels of NC (100 g/ton) were combined with either BT (200 g/ton) or CC (480 g/ton) in order to investigate potential synergistic effects and meet commercial demands for partial and complete replacement of synthetic CC, respectively. The replacement ratio used between NC and CC was 1:4.8 (1 kg of NC is equivalent to 4.8 kg of synthetic CC 60%) which was determined from previous studies[13,19].

The experimental diets (based on corn and wheat) were manufactured in the Research Diet Services BV feed factory (Hoge maat 10, 3961 NC Wijk bij Duurstede, Netherlands) and were formulated to meet the nutritional requirements for starter, grower and finisher male broilers for optimal performance (Table 2). The level of choline used in the feed formulation were equal or above NRC or primary breeder's recommendation. The other nutrients were formulated 5 to 10% below the recommendation from the genetic line[20]. All diets were completely antibiotic free, all-vegetable and formulated to have equal nutrient composition. Feed and water were provided *ad libitum*.

The phytogenic used as NC (Natu-B4™, Nuproxa Switzerland Ltd.) was composed by a blend of different parts of the plants *Solanum nigrum*, *Trachyspermum ammi* and *Achyranthes aspera*, which are sources of phosphatidylcholine and a series of phytochemicals including saponins, flavonoids, rutin and thymol. The betaine source used in this study was Betafin® S1 (Danisco Animal Nutrition), a highly purified natural source of anhydrous betaine (96%).

Data analysis: All data were first checked for normality with Quantile-Quantile plot and homogeneity of variances with a Bartlett test. The BW, FI, FCR and Average Daily Gain (ADG) were analyzed with a 2-way ANOVA over age (day of experimentation) and treatment as fixed factors, with repeated measures on the pen. In case of significance of the feed or treatments, a post-hoc procedure was conducted. A pairwise comparison for each treatment was done followed by a Tukey p-value correction. All these analyses were performed with R software version 4.1.2. Significant differences were considered when p-values were less than 0.05 and trends were considered when p-values were between 0.1 and 0.05.

RESULTS

Overall, at the end of the study (42 days) the performance of male broilers were similar or higher than the standard indicated by the genetic company (Ross 308, 2019). This indicates that even though the nutrient levels formulated in the basal diet were 5-10% below the recommended by the genetic line, the birds were able to achieve an excellent performance under the conditions of the study.

At 13 days of age, there was no difference between the treatments (data not shown). At 21 days of age, male broilers fed different treatments, including varying concentrations of NC, BT, CC and combinations of NC with BT and CC, showed no significant differences in BW, FCR, ADG or FI (Table 3). However, there was a trend for a higher FI ($p = 0.063$) in birds fed T1 (Control) and 400 g/ton of either NC or BT (T4 and T6). Similarly, there were no significant differences in BW, ADG and FI at 35 d of age (Table 4). However, at 35 days of age the FCR differed ($p = 0.026$) among treatments. Birds fed the control diet had the highest FCR, which did not differ ($p>0.05$) from broilers supplemented with the NC (100 and 200 g/ton), BT (400 g/ton), CC (480 and 960) and the combination of NC (100 g/ton) and CC (480 g/ton).

Broilers fed with BT (200 g/ton) had the most efficient feed conversion at 35 days of age, which was not different ($p>0.05$) from birds supplemented with NC (100, 200 and 400 g/ton) and broilers fed with the combination of NC (100 g/ton) and BT (200 g/ton). Overall, at 35 days of age the least efficient feed conversions were observed in male broilers fed the control diet and with CC (Table 4).

At 42 days of age, male broilers fed the different treatments did not show significant differences in average daily gain (ADG) and FI (Table 5). Nevertheless, broilers fed 400 g/ton of either BT or NC achieved the highest BW, followed by birds fed the combination of BT (200 g/ton) and CC (480 g/ton). These last three treatments were not significantly different among them for BW (Table 5). Birds fed the control feed achieved the lowest BW, which was not different from the BW of broilers fed with NC (100 and 200 g/ton), BT (200 g/ton), CC (480 and 960 g/ton) and the combination of NC (100 g/ton) and CC (480 g/ton). The FCR was also different ($p = 0.036$) between treatments at 42 days of age (Table 5). The control group, along with the birds fed with CC (480 and 960 g/ton) and the combination of NC (100 g/ton) and CC (480 g/ton), had the least efficient FCR. These treatments did not differ ($p>0.05$) among them or from birds fed with NC (100, 200 and 400 g/ton) and BT (400 g/ton). At 42 days, broilers supplemented with BT (200 g/ton) and the combination of NC (100 g/ton) and BT (200 g/ton) showed the most efficient FCR.

Table 3: Effect of the different treatments on BW, FCR, ADG and FI at 21 days of age

Treatment	BW (g)	Cum. ADG (g/b/d)	Cum. FCR	Cum. FI (g/b/d)
T1	1043±21	47.5±1.0	1.258±0.02	62.3±1.9
T2	1037±21	47.2±1.0	1.256±0.01	61.7±1.5
T3	1017±30	46.3±1.4	1.262±0.02	60.7±2.1
T4	1039±35	47.3±1.6	1.265±0.02	62.2±1.7
T5	1029±23	46.9±1.1	1.253±0.01	61.0±1.6
T6	1045±22	47.6±1.1	1.253±0.02	62.0±1.7
T7	1041±34	47.4±1.6	1.253±0.02	61.8±1.6
T8	1030±23	46.9±1.1	1.257±0.01	61.5±1.3
T9	1018±34	46.3±1.6	1.249±0.01	60.3±2.5
T10	1033±24	47.0±1.1	1.256±0.02	61.1±1.8
p-value	0.118	0.108	0.418	0.063
SEM	25	1.2	0.015	1.6

SEM: Standard error of the mean, cum: Cumulative

Table 4: Effect of the different treatments on BW, FCR, ADG and FI at 35 days of age

Treatment	BW (g)	Cum. ADG (g/b/d)	Cum. FCR	Cum. FI (g/b/d)
T1	2570±59	72.1±1.7	1.495^{a}±0.02	108.4±3.4
T2	2593±56	72.8±1.6	1.482^{abcd}±0.02	108.7±2.2
T3	2551±77	71.6±2.2	1.482^{abcd}±0.02	106.6±4.0
T4	2611±59	73.3±1.7	1.480^{bcd}±0.01	109.3±2.0
T5	2592±58	72.8±1.7	1.470^{d}±0.01	107.9±3.2
T6	2611±49	73.3±1.4	1.485^{abc}±0.02	109.5±2.9
T7	2578±56	72.4±1.6	1.490^{ab}±0.02	108.7±2.6
T8	2577±51	72.3±1.5	1.489^{ab}±0.01	108.9±2.6
T9	2585±66	72.6±1.9	1.473^{cd}±0.02	107.5±3.5
T10	2596±33	72.9±0.9	1.487^{abc}±0.02	109.1±2.5
p-value	0.299	0.295	0.026	0.331
SEM	53	1.5	0.016	2.6

$^{a-d}$Means within the same row lacking a common superscript are numerically different at p<0.05, SEM: Standard error of the mean, cum: Cumulative

Table 5: Effect of the different treatments on BW, FCR, ADG and FI at 42 days of age

Treatment	BW (g)	Cum. ADG (g/b/d)	Cum. FCR	Cum. FI (g/b/d)
T1	3438^{d}±260	80.8±1.0	1.598^{a}±0.02	128.6±3.2
T2	3471^{abcd}±240	81.6±1.2	1.587^{abc}±0.02	129.4±2.0
T3	3438^{d}±307	80.7±2.3	1.585^{abc}±0.02	127.3±4.1
T4	3500^{ab}±248	82.3±1.6	1.589^{abc}±0.02	130.0±2.2
T5	3468^{bcd}±264	81.5±1.9	1.580^{bc}±0.02	128.7±3.8
T6	3511^{a}±248	82.6±1.5	1.590^{abc}±0.02	130.5±2.6
T7	3450^{cd}±275	81.1±1.8	1.599^{a}±0.02	129.3±2.6
T8	3468^{bcd}±265	81.5±1.8	1.595^{a}±0.01	129.7±3.0
T9	3484^{abc}±292	81.9±2.0	1.576^{c}±0.02	128.5±3.0
T10	3478^{abcd}±264	81.7±1.1	1.593^{ab}±0.02	130.0±2.4
p-value	0.006	0.173	0.036	0.436
SEM	69.8	1.6	0.016	2.9

^{abcd}Means within the same row lacking a common superscript are numerically different at p<0.05, SEM: Standard error of the mean, cum: Cumulative

DISCUSSION

The study found that the birds fed both BT and NC (400 g/ton) had improved BW compared to both control and synthetic CC. Calderano *et al*.[1] also showed similar results, noting that the NC source used in this study could effectively replace CC in broiler diets with improved BW and ADG. In another study conducted by Jadhav *et al*.[21] the authors observed that, supplementing this same source of NC led to improved BW and ADG compared to CC and a choline-deficient diet.

The average FI and ADG (g/b/d) of male broilers during the study did not differ (p>0.05) between treatments. These results indicate that the choline requirement for these parameters was fulfilled by the choline contained in the feedstuffs (i.e., corn, wheat and soybean meal) used in the experimental feeds and for the conditions of this particular study. This finding is supported by the study of Pesti *et al*.[22] who reported that a diet based on corn and soybean-meal contained approximately 1,350 mg choline kg^{-1} and the study of Molitoris and Baker[23] who indicated that this level was enough to meet the dietary requirement of broiler chickens. The results of this study showed that a more sensitive indicator of the influence of the dietary treatments on broiler performance was the FCR. Overall, the results at 35 and 42 days show that the least efficient FCR was observed in the control birds. At 42 days, the only treatments that differ (p>0.05) from the control, achieving a better FCR were the birds supplemented with BT (200 g/ton) and the combination of NC (100 g/ton) and BT (200 g/ton). This finding somehow aligns with the study of Khose *et al*.[15] where treatment groups supplemented with NC exhibited improved FCR compared to the non-supplemented choline group (control).

When comparing the different sources of methyl groups evaluated in this study, we concluded that based on FCR, the NC performed similarly, independent of the dose evaluated (100-400 g/ton). In addition, no differences (p>0.05) were observed for broilers fed NC and BT, independent of the supplemented dose. The most efficient feed conversion at 42 days was observed in male broilers fed the combination of NC (100 g/ton) and BT (200 g/ton). This finding may suggest a synergistic effect of both additives that deserves further research. The BT is a methyl group donor for the methylation cycle and the NC tested in this study supplies both a highly available source of choline and a series of phytochemicals with lipotropic activity. Previous study has shown that the NC used in the present study increased the expression of PPARα in the liver and adiponectin in the blood of broilers[16]. These findings may suggest that NC better supports the lipotropic action of choline while BT the methyl group donor activity for the methylation cycle, supporting each other function in the metabolism.

Another important finding of this study is that male broilers fed with the NC achieved similar or better performance than the birds fed with CC, indicating that the NC could safely replace CC in broiler feeds. These findings agree with previous study conducted by Petrolli *et al.*[17] who showed that replacing CC with NC in male broiler diets decreases FCR and maintains BW, suggesting that NC can effectively replace CC, improving overall performance. In fact, there were no significant differences in performance of male broilers at 21, 35 or 42 days of age fed either 100 g/ton of the NC or 960 g/ton of CC 60%.

We concluded that regardless of the level of synthetic CC and NC in the diets, broiler growth remained consistent, with some cases showing improved zootechnical performance when supplementing a combination of NC and BT. Similarly, Khose *et al.*[15] reported that supplementing the same NC in broiler diets could replace synthetic CC without affecting zootechnical performances. This supports the idea that CC can be safely replaced with alternative sources, including selected plants, such as the combination of *Solanum nigrum*, *Trachyspermum ammi* and *Achyranthes aspera*, BT or with combination of NC and BT.

CONCLUSION

This study highlighted the potential of natural choline from plant sources (NC) and BT as suitable replacements for CC in broiler diets, maintained zootechnical performance while provided several benefits. The low inclusion rate of NC and BT as CC replacements not only maintained feed efficiency but also allowed greater flexibility in feed formulation by incorporating cheaper ingredients without compromising quality. In addition, the potential synergistic effect observed between NC and BT, particularly in older broilers, suggested a promising direction for further investigation of their combined effects on energy metabolism. These findings have significant implications for the poultry industry, providing a sustainable and cost-effective alternative to synthetic additives. Future research should explore the commercial application of these substitutes in more detail, investigate additional zootechnical performance indicators and conduct comprehensive economic analyses to determine the return on investment associated with substituting CC in broiler diets.

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