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Method of Production and Assessment of an Encapsulated Choline Chloride and Its Effects on Growth Performance and Serum Lipid Indices in Broilers

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Abstract

Since, choline chloride caking causes serious operating difficulties and customer complaints, two experiments were conducted to optimize *in vitro* production of a novel encapsulated choline chloride (ECC) with minimum hygroscopic property and optimize delivery in gastrointestinal tract (GIT). The *in vivo* verification test of ECC was used to compare it with the commercial choline chloride (CC) in Ross 308 broiler chickens. Twelve factors with 3 levels including 27 formulations on ECC properties were evaluated using the Taguchi method (signal/noise ratio analysis). The produced ECC particles showed a decrease in hygroscopic property and release rate under simulated GIT. The *in vitro* study showed that the encapsulation efficiency of 27 formulations were ≥ 80% and choline content in ECC particles ranged from 507 to 718 g/kg (wt/wt). The oil, wax, whey protein concentrate (WPC), and calcium stearate contents had the most influence on hygroscopic property ($P < 0.05$). The ECC particle gastric resistance was improved by increasing oil and wax contents as well as sonication time, pH, and carrier content ($P < 0.05$). Average daily gain of broiler chickens fed diet supplemented with choline chloride (CC or ECC) was increased compared to those fed negative control diet during starter period ($P < 0.05$). The serum concentration of low-density lipoprotein-cholesterol, triglyceride, and cholesterol were decreased in birds fed diet supplemented with choline chloride (CC or ECC; $P < 0.05$). The results showed that ECC with no hygroscopic property might be an alternative to CC without negative effect on performance of broiler chickens.

Introduction

Agglomeration is a natural phenomenon in preservation and processing of feed manufacturing. Unwanted agglomeration typically called caking, refers to the state of a bulk solid material that has lumped or agglomerated because of strong bonds between particles. Usually caking of feed or ingredient causes serious operating difficulties and customer complaints. The main mechanisms of bond forming in particles are mechanical interlocking, plastic-flow bonding, electrostatic charging, physio-chemical bonding including moisture caking, and combination of these factors (Griffith, 1991). Moisture caking is the most common caking mechanism in the bulk solid industries affecting feedstuff/food stuff and crystalline powder system. Environmental factors in storage condition including humidity, temperature, time, and along with pressure affect moisture caking (Barbosa- Canovas *et al*., 2005).

Among the various methods of feed processing, encapsulation may help to prevent or reduce moisture absorption and caking in hygroscopic materials (Shahidi and Han, 1993). Microencapsulation is an advanced feed technology that has increasing attention in animal nutrition. This technology protects the sensitive compounds (such as feed/food ingredients, vitamins, amino acids, organic acids, enzymes, cells or other materials) during feed processing and storage conditions, maintaining nutrient bioavailability (Shahidi and Han, 1993).

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In animal nutrition, choline chloride is the most hygroscopic additive with high potential to caking. Due to hygroscopic nature of choline chloride, it transforms to cake form in ambient condition. Caked choline chloride cannot be mixed with other feed/food ingredients properly and it also reduces the bioavailability of other vitamins (Barbosa- Canovas *et al*., 2005). The objective of this study was to achieve optimum formula for encapsulation of choline chloride to protect choline from undesirable particle moisture absorption in ambient condition and also optimize release rate in the upper and lower gastrointestinal tract (GIT) of the chickens.

Materials and Methods

Description of study materials

The experimental procedures were approved by the Ferdowsi University of Mashhad Animal Care and Use Committee (Protocol No. 29818). Choline chloride was encapsulated in the Research Institute of Food Science and Technology (Mashhad, Iran) and was approved by the Intellectual Property Center of Iran (Patent Number: 90158).

Microcapsule core materials included liquid choline chloride (750 mg/g) was obtained from NB Group Ltd., China. Commercial choline chloride (CC) supplied from Nutrex Company, Belgium. Pepsin and Pancreatin were purchased from Sigma-Aldrich.

In vitro Experimental Design

Twelve factors with 3 levels were tested as independent variables (Table 1). Taguchi L27 (3^{12}) orthogonal array method was used to systematically assess the effects of independent variables on encapsulated choline chloride (ECC) particle properties including release rate in simulated gastric fluid (SGF) and simulated intestinal fluids (SIF), hygroscopic property (moisture absorption), and angle of repose. The 27 designed experimental treatments found from Taguchi orthogonal array are shown in Table 2. To identify the approximate range of each factor, preliminary experiments were conducted.

Table 1. Twelve factors and three levels involved in encapsulated choline chloride production

	Factors											
Levels	Carrier size (µm)	Carrier content (g/kg)	LCC[1] pH	LCC[1] Temperature	Calcium Stearate (g/kg)	Core size (µm)	Core content (g/kg)	Oil Source	Oil content (g/kg)	Wax content (g/kg)	WPC[2] concentration (g/kg)	Sonication Time (min)
1	45 to 63	200	8	60	30	125 to 500	100	Stearic acid	5	0	40	2
2	63 to 125	250	10	75	50	500 to 1000	125	Soy oil	15	5	80	3
3	125 to 250	300	12	90	70	1000 to 2000	150	Palm oil	25	10	120	4

[1]Liquid choline chloride (purity 75%; NB Group Ltd., China)
[2]Whey protein concentrate

Encapsulated choline chloride production

The selected factors for core production were carrier size (µm), carrier content (g/kg), pH, temperature, and binder content (g/kg). At first, carrier (bentonite) was sieved by auto-stirred sieve (AS 400 control, Retsch, Germany) and 3 particle sizes were separated (45-63, 63-125 and 125-250 µm). The pH of liquid choline chloride was adjusted by NaOH (2 mol/L) to 8, 10, and 12. Temperature of choline chloride was set at 60, 75, and 90ºC. In order to improve mixability the bentonite was mixed with calcium stearate and then liquid choline chloride (purity 75%; NB Group Ltd., China) was added to the mixture. This solution was stirred for 60 min by a magnetic stirring (Ika RCT basic, Germany). Samples were oven-dried (FP53, Binder Co., Germany) at 75ºC for 24 h and then were grinded (A11, Ika Co., Germany) and sieved by auto-stirrer sieve. In the next step, samples were separated according to 3 core particle sizes (125 to 500, 500 to 1000 and 1000 to 2000 µm). The sample formulations were prepared in final weight of 100 g and choline chloride content was adjusted based on 100% purity. In wall synthesis phase, seven parameters were evaluated including core size, core content, preliminary wall materials, oil or fatty acid content, secondary wall materials (bee wax) content, WPC levels and sonication time.

To synthesis the wall particles, hydrogenated soy, palm oils, and stearic acid were melted and heated in 75ºC. Whey protein concentrate 20 g in 80 mL water (2:8 WPC: Water as stock solution) was also dispersed in deionized water and stirred (500 *rpm*) for 1 h at room temperature and adjusted to pH 8 with 2 mol/L of NaOH. Whey protein concentrate stock

solution was left overnight for hydration. The melted preliminary (soy oil, palm oil and stearic acid) and secondary walls (bee wax) were sprayed on core particles, respectively, and mixed for 5 min. Whey protein concentrates were made at different levels including 40, 80, and 120 g/kg and immediately magnetic stirred to denature protein for 30 min (Ika RCT basic, Germany) in 75ºC and subsequently emulsified using probe sonication for 2, 3, and 4 min (VCX750, Sonics, UK) with 99% amplitude to denature protein. Emulsion was discharged in aluminum dish and heated 24 h in 60ºC for moisture reduction (FP53, Binder Co. Germany). To produce EC, dried samples were milled and finally 1000-2000 µm particle sizes were separated.

Table 2. Compositions of the 27 *in-vitro* formulations used for optimization of encapsulated choline chloride.

	Factors											
Formulation	Carrier size (µm)	Carrier content (g/kg)	LCC[1]pH	LCC Temperature	Calcium tearate (g/kg)	Core size (µm)	Core content (g/kg)	Oil Source	Oil content (g/kg)	Wax content (g/kg)	WPC[2] concentration (g/kg)	Sonication Time (min)
1	45 to 63	250	8	60	30	125 to 500	100	Stearic acid	5	0	40	2
2	45 to 63	250	8	60	50	500 to 1000	125	Soy oil	15	5	80	3
3	45 to 63	250	8	60	70	1000 to 2000	150	Palm oil	25	10	120	4
4	45 to 63	300	10	75	30	125 to 500	100	Soy oil	15	5	120	4
5	45 to 63	300	10	75	50	500 to 1000	125	Palm oil	25	10	40	2
6	45 to 63	300	10	75	70	1000 to 2000	150	Stearic acid	5	0	80	3
7	45 to 63	200	12	90	30	125 to 500	100	Palm oil	25	10	80	3
8	45 to 63	200	12	90	50	500 to 1000	125	Stearic acid	5	0	120	4
9	45 to 63	200	12	90	70	1000 to 2000	150	Soy oil	15	5	40	2
10	63 to 125	250	10	90	30	1000 to 2000	100	Stearic acid	15	10	40	3
11	63 to 125	250	10	90	50	125 to 500	125	Soy oil	25	0	80	4
12	63 to 125	250	10	90	70	500 to 1000	150	Palm oil	5	5	120	2
13	63 to 125	300	12	60	30	1000 to 2000	100	Soy oil	25	0	120	2
14	63 to 125	300	12	60	50	125 to 500	125	Palm oil	5	5	40	3
15	63 to 125	300	12	60	70	500 to 1000	150	Stearic acid	15	10	80	4
16	63 to 125	200	8	75	30	1000 to 2000	100	Palm oil	5	5	80	4
17	63 to 125	200	8	75	50	125 to 500	125	Stearic acid	15	10	120	2
18	63 to 125	200	8	75	70	500 to 1000	150	Soy oil	25	0	40	3
19	125 to 250	250	12	75	30	500 to 1000	100	Stearic acid	25	5	40	4
20	125 to 250	250	12	75	50	1000 to 2000	125	Soy oil	5	10	80	2
21	125 to 250	250	12	75	70	125 to 500	150	Palm oil	15	0	120	3
22	125 to 250	300	8	90	30	500 to 1000	100	Soy oil	05	10	120	3
23	125 to 250	300	8	90	50	1000 to 2000	125	Palm oil	15	0	40	4
24	125 to 250	300	8	90	70	125 to 500	150	Stearic acid	25	5	80	2
25	125 to 250	200	10	60	30	500 to 1000	100	Palm oil	15	0	80	2
26	125 to 250	200	10	60	50	1000 to 2000	125	Stearic acid	25	5	120	3
27	125 to 250	200	10	60	70	125 to 500	150	Soy oil	05	10	40	4

[1]Liquid choline chloride (purity 75%; NB Group Ltd., China)
[2]Whey protein concentrate

In vitro Characterization of the Particles

Moisture Content

The ECC samples (5 g) were oven-dried at 105ºC for 3 h (FP53, Binder Co., Germany) and weight differences of the samples before and after drying were considered as particle moisture content (g/kg).

Moisture Absorption

The moisture absorption was measured by weighing 1 g of sample before and after incubation at 25ºC and 50% humidity (KBWF 240, Binder, Germany). Sample weights were recorded during 24 h (at h 1, 2, 3, 4, 5, 6, 9, 12, and 24) based on weight changes.

Angle of Repose

The angle of repose is the angle made by the horizontal base of the flat surface and the edge of a cone-like pile of granules. (Barbosa- Canovas *et al.* 2005). The height of heap above the floor and the diameter of the heap at its base were determined and the angle of repose (φ) was measured by the following equation:

Angle of Repose ϕ (°) $= tan^{-1}$ 2h/D

where, Φ = angle of repose (◦); h = height of the pile (mm); D = diameter of the pile (mm).

Choline Content Determination

To determine the choline content of particle surface, 0.1 g of ECC was dispersed in 1 mL of deionized water and vortexed for 30 seconds. The sample mixture was centrifuged at 7000 g for 10 min at 20ºC (2-16-Plc, Sigma, Germany). Supernatant was removed and choline content determined by HPLC (Tao *et al.*, 1984).

The encapsulation efficiency (EE) was determined as follows:

EE (%) = [(g choline initially taken to prepare the particle - g choline content on particle surface)/ g choline initially taken to prepare the particle] × 100

Release Rate of Choline from Encapsulated Particle

The gastric resistance and release rate of encapsulated choline chloride were investigated in SGF and SIF (USPCCE, 2004). The SGF was composed by dissolving 3.2 g/L of pepsin in 2 g/L of NaCl solution at pH 1.5. Simulated intestinal Fluid was made of 10 g/L of pancreatin and 0.05 mol/L of KH2PO4 at pH 7.4. Encapsulated choline chloride sample (0.1 g) was suspended into 9 mL of SGF. The mixture was set for 60 min in auto-shaker incubator (D38678, GFI Co., Germany) at 37ºC and 300 *rpm*. Then, samples were centrifuged (2-16-Plc, Sigma Co., Germany) at 2,316.2 g for 30 min at 20ºC. The supernatant was separated and choline content was measured by HPLC method accordingly (Tao *et al.*, 1984). To obtain release rate in intestinal segment, 17 mL of SIF was appended to the pellet and incubated for 120 min in shaking rate of 300 *rpm* at 37ºC. During incubation 4 tubes from each sample were taken at 30, 60, 90, and 120 min intervals and centrifuged (1000 g, 15 min, 4ºC) to obtain supernatant.

Comparison of Encapsulated and Commercial Choline Chloride

Optimum formulation of ECC with the best flowability (minimum moisture absorption and angle of repose) and optimum release rate was measured and then ECC was compared with CC in an *in vivo* experiment.

Table 3. Ingredients and nutrient compositions of the basal diets (g/kg as fed)[1]

Ingredients	Starter (1 – 10 d)	Grower (11-24 d)
Corn (78 g/kg CP)	520.0	570.0
Soybean meal (440 g/kg CP)	305.0	267.0
Corn gluten meal (600 g/kg CP)	80.0	78.0
Wheat bran	10.0	0.0
Vegetable oil	36.0	40.0
Limestone	15.0	14.0
Dicalcium phosphate	15.5	14.0
Sodium chloride	3.5	3.0
DL_Methionine	2.5	2.0
L-Lysine-HCL	4.0	4.0
L-Threonine	0.5	0.0
Vitamin premix[2]	2.5	2.5
Mineral premix[3]	2.5	2.5
Sand	3.0	3.0
Total	1000	1000
Calculated Analysis (g/kg)		
ME (kcal/kg)	2995	3085
Protein	230.6	214.7
Calcium	9.1	8.7
Available phosphor	4.6	4.4
Sodium	1.8	1.8
Lysine	12.8	11.8
Methionine	6.65	5.9
Methionine + Cystine	9.5	8.7
Threonine	8.7	7.7
Arginine	12.9	11.9
Tryptophan	2.2	1.9

[1]The other 4 diets were generated by adding two sources of choline chloride (commercial and encapsulated) at the rate of 800 and 1700 mg/kg to the basal diet and were designated as treatment 2, 3, 4 and 5, respectively.

[2]Vitamin premix contained the following per kilogram of diet: vitamin A, 16,000 IU; vitamin D3, 3,000 IU; vitamin E, 150 mg; vitamin B1, 3 mg; vitamin B2, 10 mg; vitamin B6, 3 mg; biotin, 7.5 mg; vitamin B12, 15 µg; nicotinic acid, 60 mg; pantothenic acid, 14.7 mg; folic acid, 1.5 mg.

[3]Mineral premix contained the following per kilogram of diet: iron, 20 mg; copper, 10 mg; manganese, 100 mg; cobalt, 1.0 mg; zinc, 82.2 mg; iodine, 1 mg; selenium, 0.2 mg; molybdenum, 0.5 mg.

Birds and Management

A total of 360 one-d-old straight-run broiler chicks (Ross 308) were randomly allocated to 5 dietary treatments with 6 replicates of 12 chicks each, in a completely randomized design. Corn-soybean meal-based diet (mash form) without choline was used as negative control. The other 4 diets were supplemented with 800 to 1700 mg choline chloride (CC and ECC) per kg of diet (Table 3). The feeding program consisted of starter and grower diets fed from 1-10 and 11-24 d of age, respectively. Lighting program was 23L:1D throughout the experiment. The room temperature was 32°C on d 1 and then was gradually reduced to reach 25°C by 24 d of age and remained constant thereafter. Body weight (BW) and feed intake (FI) of the birds was recorded for starter and grower periods and then average daily gain (ADG) and average daily feed intake (ADFI) were calculated. The FCR was calculated by dividing ADG to ADFI (Bakhshalinejad *et al.*, 2018).

Blood Characteristics

Three chickens were randomly selected from each replicate and blood samples were collected from the brachial vein into the heparinized tubes on d 24. Sera samples were used to measure the concentration of triglyceride (TG), cholesterol (CHOL), low-density lipoprotein-cholestrol (LDL-c) and high-density lipoprotein-cholestrol (HDL-c) by an automatic biochemical analyzer (A15 Biosystems, Co. Spain) (Bakhshalinejad *et al.*, 2018).

Statistical Analysis

A Taguchi method of L27 (3^{12}) consisted of 12 variables with 3 levels was used, which known as the signal to noise (S/N) ratio (Table 1). Data were analyzed by Minitab 17 software (Minitab, 2014). The means and variances of each response at each setting of factors in orthogonal array were combined into a single performance in the Taguchi platform to search a combination of factors with appropriate levels that gives optimal response. The S/N equation for optimal response depends on the pattern of the quality characteristic. The selected L27 (3^{12}) Taguchi orthogonal arrays are shown in Table 2. According to the types of characteristics, the S/N ratios vary and can be determined as equation 1 and 2 for the 'smaller is better' and 'larger is better' number of characteristics, respectively:

$$\text{Equation 1; smaller is better } S/N = -10log\left(1/n\sum_{i=0}^{n} yi^2\right)$$

$$\text{Equation 2; larger is better } S/N = -10log\left(1/n\sum_{i=0}^{n} 1/yi^2\right)$$

Where, S/N is the signal to noise ratio, *n* is the total number of experiments in the orthogonal array and *yi* is the *ith* data obtained (Sedghi *et al.*, 2014).

In vivo data were analyzed by the General Linear Model (GLM) procedure of SAS software (SAS, 2003). Means were compared using Duncan's multiple comparison tests when diet effect was significant ($P < 0.05$). Orthogonal polynomial contrasts were used to test the effects of choline supplementation. All percentage values were transformed using arc sine before analysis.

Results

***In Vitro* Characteristics**

Encapsulation Efficiency

Encapsulation efficiency was more than 80% in all production formulas (Table 4). The choline chloride content (wt/wt) and surface choline chloride content (wt/wt) of the capsules in all formulations were ranged from 507 to 718 g/kg and 125 to 213 g/kg, respectively. The ECC in capsule particles was 605 g/kg choline chloride (wt/wt).

Moisture Content

In all formulations, the moisture content ranged from 57 to 109 g/kg depending on each case (Table 4). The ECC moisture content was 91 g/kg.

Hygroscopic Property

Moisture absorption in all formulations measured at 1, 2, 3, 4, 5, 6, 9, 12 and 24 h that was become constant at h 12 (Table 4). The equilibrium moisture contents of choline chloride particles in all formulation ranged from 82 to 191 g/kg based on dry matter in 12h and for ECC, it was 131 g/kg. The oil, wax, WPC, and calcium stearate content had the great impact on moisture absorption content and subsequently hygroscopic property of ECC based on analysis of S/N ratios (Table 4).

Angle of Repose

The most effective factor on angle of repose was carrier content (Table 4), where the angle of repose was decreased with increasing carrier content. The angle of repose was lower in particles with minimum moisture content and it was ranged from 38° to 53° in choline chloride particles among formulations. The ECC angle of repose was 42° (Table 4).

Release Rate in SGF

The choline chloride release rates were in the range of 135 to 210 g/kg among 27 formulations. The release rate of ECC in SGF was 130 g/kg (Table 4). Particle gastric resistance was increased by increasing oil (stearic acid and soy oils) and wax contents in outer layers. Sonication time, pH, and carrier content had remarkable effect on gastric resistance of particles. Release rate of choline chloride was reduced with increase in core size, choline chloride temperature, and whey protein concentrations.

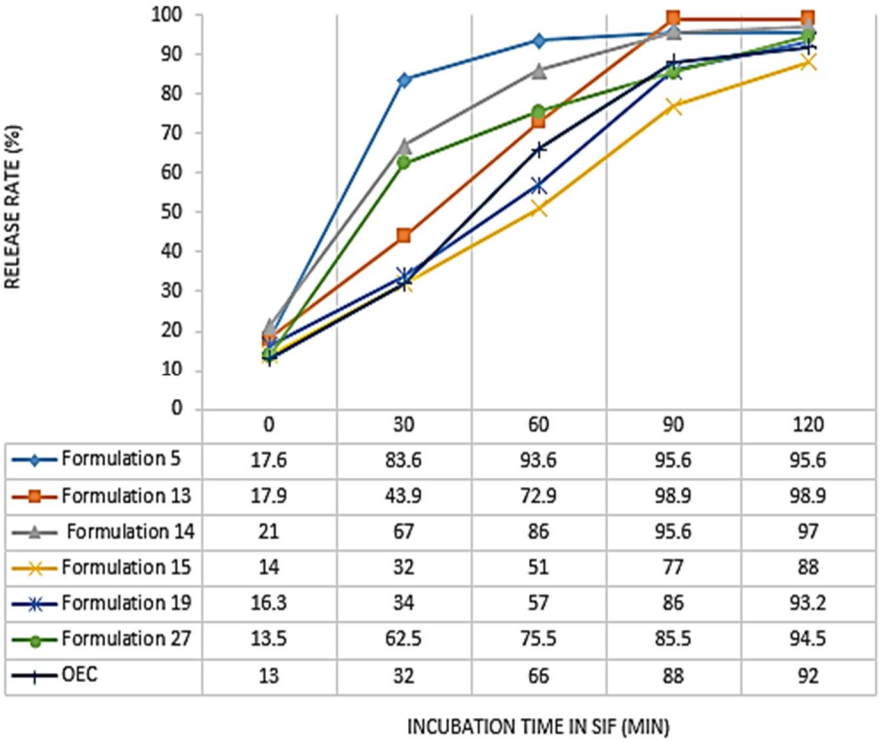
Table 4. Specifications of the encapsulated choline chloride productions from different formulations.

Formulations	Surface choline (g/kg)	Choline content (g/kg)	Encapsulation Efficiency (g/kg)	Moisture content (g/kg)	Moisture absorption at h 12 (g/kg)	Angle of Repose[1] (φ)	Release rate in SGF (g/kg)
1	156	684	844	88	171	47	192
2	125	602	875	89	115	53	155
3	213	550	787	71	111	48	160
4	132	556	868	98	100	48	157
5	166	507	834	68	93	46	176
6	195	608	805	91	153	39	185
7	145	569	855	70	980	41	171
8	191	718	809	95	169	47	169
9	140	643	860	88	179	46	166
10	133	575	867	73	133	51	180
11	139	582	861	78	116	53	190
12	170	635	830	87	144	53	182
13	155	533	845	85	133	50	179
14	190	601	810	99	149	50	210
15	149	539	851	105	96	43	140
16	201	697	799	96	117	39	190
17	185	623	815	79	122	38	150
18	180	625	820	86	157	41	170
19	144	553	856	57	109	50	163
20	195	623	805	83	159	46	190
21	178	616	822	80	147	48	188
22	164	580	836	109	82	47	170
23	174	580	826	83	135	42	189
24	152	524	848	58	118	44	173
25	179	667	821	58	131	39	181
26	134	603	866	87	116	41	172
27	181	663	819	74	191	41	135
ECC[2]	149	606	851	91	131	42	130
CC[3]	ND[4]	156	ND	55	285	39	685
S/N Ratio[5]	37.746	-20.058	39.242	-13.503	-16.005	-30.282	-21.527
POF Means[6]	77	86.8	913	38.7	40.9	30.666	110.7
			P-values				
Carrier size (μ)	ND	0.909	0.909	0.483	0.955	0.169	0.694
Carrier content	ND	0.730	0.730	0.527	0.206	0.015	0.444
pH	ND	0.573	0.573	0.767	0.515	0.148	0.915
Temperature (ºC)	ND	0.410	0.410	0.958	0.799	0.108	0.642
Calcium Stearate	ND	0.466	0.466	0.925	0.210	0.371	0.407
Core Content	ND	0.600	0.160	0.622	0.759	0.266	0.899
Core Size (μ)	ND	0.711	0.711	0.876	0.370	0.215	0.423
Oil (Source)	ND	0.285	0.285	0.582	0.532	0.117	0.267
Oil content	ND	0.213	0.213	0.256	0.138	0.710	0.371
Wax	ND	0.391	0.391	0.934	0.193	0.114	0.217
WPC	ND	0.846	0.846	0.591	0.191	0.139	0.699
Sonication Time(min)	ND	0.747	0.747	0.521	0.481	0.941	0.368

[1]Angles of up to 35º indicate free flowability, 35º–45º some cohesiveness, 45º –55º cohesiveness (loss of free flowability), and 55º and above very high cohesiveness (very limited or no flowability) (Carr, 1976)
[2]encapsulated choline chloride
[3]Commercial choline chloride that was tested in 4 replications
[4]Not detected
[5]Signal to noise ratio
[6]The mean of predicted optimum formula by the Minitab 17 software (Stat/DOE/Taguchi/predict Taguchi results)

Release Rate in SIF

The release properties of ECC evaluated in simulated intestinal fluid (SIF). Particle release rate of all 27 formulations were varied and released up to 770 g/kg after 90 min incubation in SIF. Release profile of test samples and ECC are represented in Figure. The optimum intestinal release of choline chloride was mainly due to their high percentage of hydrogenated soy oil or stearic acid and wax in preliminary and secondary outer layers (walls), respectively. Encapsulated particles with palm oil in outer wall had the weakest resistance in SIF in comparison to those of other oil layers (Table 4).



	0	30	60	90	120
Formulation 5	17.6	83.6	93.6	95.6	95.6
Formulation 13	17.9	43.9	72.9	98.9	98.9
Formulation 14	21	67	86	95.6	97
Formulation 15	14	32	51	77	88
Formulation 19	16.3	34	57	86	93.2
Formulation 27	13.5	62.5	75.5	85.5	94.5
OEC	13	32	66	88	92

Figure 1. Release profile of choline particles from selected formulations of encapsulated choline chloride. The optimum formulation of encapsulated choline chloride (OEC) was obtained via *in vitro* optimization procedures in simulated intestinal fluid (SIF)

The regression equation obtained from the ECC release rate in SIF (min 60) was as follows:
82.993+7.130 carrier $size_1$-4.648 carrier $size_2$-2.481 carrier $size_3$ +1.785 carrier $content_1$ -0.615 carrier $content_2$ -1.170 carrier $content_3$ +0.607 pH_1+1.874 pH_2-2.481 pH_3-6.948 Tem_1+ 3.219 Tem_2+ 3.730 Tem_3+ 0.596 calcium $stearate_1$+1.241 calcium $stearate_2$- 1.837 calcium $stearate_3$+0.263 core $content_1$-0.993 core $content_2$ +0.730 core $content_3$+2.685 core $size_1$ -5.370 core $size_2$+2.685 core $size_3$ -5.115 oil $source_1$ +0.141 oil $source_2$ +4.974 oil $source_3$ +5.263 oil $content_1$ -3.481 oil $content_2$ -1.781 oil $content_3$+2.930 wax_1 +1.152 wax_2 -4.081 wax_3 +2.241 WPC_1-1.993 WPC_2-0.248 WPC_3 +2.163 $sonication_1$ -0.870 $sonication_2$- 1.293 $sonication_3$.

The comparison results of ECC *vs.* CC results are shown in Table 4. The moisture content and moisture absorption (12h) for CC and ECC were 57, 285 g/kg and 91, 131 g/kg, respectively. The angle of repose and release rate in SGF for CC was 39° and 685 g/kg, respectively. The HPLC analyses of choline content for ECC, CC, and liquid choline chloride were 593, 610, and 770 g/kg, respectively.

In Vivo Experiment

Performance

Calculated and analyzed content of choline chloride in experimental diets are shown in Table 5. The birds remained healthy throughout the trial period and the percentage of mortality was negligible among all treatments.

The effects of ECC and CC supplementation on growth performance of broiler chickens are shown in Table 6. There was not a significant effect on growth performance of the birds fed supplemental levels of ECC and CC during 11-24 d of age. Although ADFI and FCR were not affected by choline supplementation, birds fed negative control diet had lower ADG than those fed diets supplemented with CC in the starter period ($P < 0.05$). Orthogonal contrast revealed that the source of choline chloride had no significant effect on growth performance of the chickens (Table 6).

Blood Characteristics

Effect of ECC and CC supplementation on serum biochemical attributes of broiler chickens are shown in Table 7. The concentrations of CHOL, TG, and LDL-c in sera were significantly decreased in birds fed diets supplemented with choline chloride ($P < 0.05$). Based on orthogonal contrasts, TG content in chickens fed diets containing ECC was higher than those fed diet supplemented with CC (42.9 *vs.* 36.9 mg/dL; $P < 0.05$), whereas CHOL value was not influenced by the type of choline chloride sources ($P > 0.05$).

Table 5. Calculated and analyzed content of choline chloride in experimental diets.

	Choline chloride (mg/kg diet)	
Diets	Calculated	Analyzed
NC[1]	900	1025
NC+ 800 mg/kg CC[2]	1700	1620
NC+ 1700 mg/kg CC	2600	2710
NC+ 800 mg/kg ECC[3]	1700	1840
NC+ 1700 mg/kg ECC	2600	2850

[1]Negative control diet without choline chloride supplementation
[2]Commercial choline chloride
[3]Encapsulated choline chloride

Table 6. Effects of diet supplemented with encapsulated and commercial choline chloride at different levels on growth performance in broiler chickens[1]

Items	Starter (1-10 d)			Grower (11-24 d)			Overall (1-24 d)		
	ADFI[2] (g/b/d)	ADG[3] (g/b/d)	FCR[4]	ADFI (g/b/d)	ADG (g/b/d)	FCR	ADFI (g/b/d)	ADG (g/b/d)	FCR
NC[5]	21.0	17.3[b]	1.235	74.8	51.8	1.441	52.3	37.4	1.405
NC+ 800 mg/kg CC[6]	22.2	19.2[a]	1.152	75.8	52.0	1.459	53.5	38.4	1.395
NC+ 1700 mg/kg CC	21.8	18.8[a]	1.160	76.3	50.7	1.501	53.6	37.4	1.433
NC+ 800 mg/kg ECC[7]	22.0	19.2[a]	1.147	76.7	52.1	1.476	53.8	38.5	1.410
NC+ 1700 mg/kg ECC	22.0	18.7[a]	1.175	75.7	51.8	1.465	53.3	38.0	1.405
SEM[8]	0.59	0.212	0.029	1.29	0.80	0.074	0.83	0.491	0.020
Orthogonal Contrast	*P*-value for contrasts								
CC *vs.* ECC	0.980	0.816	0.847	0.903	0.455	0.581	0.909	0.509	0.753
NC *vs.* CC+ECC	0.131	<0.001	0.031	0.368	0.925	0.201	0.212	0.225	0.805
NC *vs.* CC	0.168	<0.001	0.040	0.438	0.695	0.166	0.272	0.387	0.724
NC *vs.* ECC	0.165	<0.001	0.055	0.384	0.824	0.341	0.235	0.176	0.923

[a-b] Means within a column without a common superscript differ significantly ($P < 0.05$).
[1]All means are average of 6 pens per treatment
[2]ADFI = average daily feed intake
[3]ADG=average daily gain
[4]FCR=feed conversion ratio
[5]Negative control diet without choline chloride supplementation
[6]Commercial choline chloride
[7]encapsulated choline chloride
[8]Standard error of mean

Table 7. Effects of diet supplemented with encapsulated and commercial choline chloride at different levels on serum chemistry (mg/dL) at 24 d of age in broiler chickens[1]

Items	CHOL[2]	TG[3]	HDL-c[4]	LDL-c[5]
NC[6]	83.17[a]	56.57[a]	71.45	14.79[a]
NC+ 800 mg/kg CC[7]	59.40[ab]	37.41[ab]	52.65	4.41[b]
NC+ 1700 mg/kg CC	56.49[b]	36.37[b]	55.84	7.39[b]
NC+800 mg/kg ECC[8]	70.26[ab]	46.91[ab]	70.22	7.80[b]
NC+1700mg/kg ECC	75.86[ab]	38.90[ab]	47.92	5.90[b]
SEM[9]	8.297	8.746	7.998	2.121
Orthogonal Contrast	*P*-value for contrasts			
CC *vs.* ECC	0.067	0.022	0.550	0.745
NC *vs.* CC+ECC	0.090	0.089	0.147	0.004
NC *vs.* CC	0.027	0.046	0.120	0.006
NC *vs.* ECC	0.368	0.252	0.256	0.010

[a-b] Means within a column without a common superscript differ significantly ($P< 0.05$).
[1]All means are average of 18 birds per treatment.
[2]Cholesterol
[3]Triglyceride
[4]High-density lipoprotein-cholesterol
[5]Low-density lipoprotein-cholesterol
[6]Negative control diet without choline chloride supplementation
[7]Commercial choline chloride
[8]Encapsulated choline chloride
[9]Standard error of mean

Discussion

The production of a free-flowing choline chloride through encapsulation process was one of the main targets of this study. Previous production methods had two disadvantages including; expensive equipment and lack of improved flowability (Kiefer *et al.*, 1996, Mehta, 2005, Eversdijk *et al.*, 2013). Caking in premixes during high shelf temperature and moisture would decrease the quality of vitamins and mineral supplements (Roudaut, 2008).

In this study, moisture absorption of ECC was reduced by 550 g/kg compared to CC and flowability was improved as identified by angle of repose (42°). Angle of repose is considered as a common method to measure flow properties (Craik and Miller, 1958). More flowable material has less angle of repose (Carr, 1965). The adhesion (Craik and Miller, 1958) and cohesion (Moreyra and Peleg, 1981) in a powder increase when its moisture content increases.

Statistical analysis of the above model revealed that carrier size, core size, choline temperature, oil source, oil content and wax content had the most pronounced influence on the encapsulated choline chloride release rate in SIF. In addition, S/N ratios of Taguchi method revealed that WPC in 80 g/kg level had the best release rate in SIF. Changes of WPC level could change particle resistance.

The most outcome of this study was the protection of choline chloride in alkaline solution that could improve the choline quality in the carrier. Chandler (1996) stated that alkali solution could penetrate into the carrier pores, making soap caps on the surface of pores via chemical reactions with fatty acids. Implementation of fatty acids in supplement formulations as alkali metal soaps such as calcium stearate improved the plasticity property of encapsulated choline chloride (Chandler, 1996). In the current study, it is hypothesized that this fatty acid matrix in outer layer of encapsulated choline chloride might optimize the release rate of choline compound in GIT.

The source of choline chloride (ECC or CC) did not affect the growth performance of broiler chickens during 24 d of experiment, but increased ADG of the birds during the starter period. Younger chickens have a higher requirement for choline than older birds (NRC, 1994) that may indicates higher needs to methyl donor groups in early than latter growing periods. In other word, *de novo* biosynthesis of choline may increase with age in chickens (NRC, 1994). Derilo and Balnave (1980) demonstrated that dietary choline supplementation by 571, 871, 1171, 1471, and 1771 mg/kg of the diet significantly increased ADG and improved FCR in young broiler chicks, which is in agreement with the results of this study.

Dietary requirement of choline depends on the level of other nutrients involve in methyl group metabolism (NRC, 1994). During rapid growth, the high sensitivity to the marginal levels of methionine may occur if dietary choline is not sufficient (Blair *et al.*, 1986). Methionine and betaine (3 methyl glycine, used as methyl donor) levels in experimental diets were sufficient in this study and might be the reason for similar growth response in broiler chickens fed diets supplemented with different levels of choline chloride during 11 to 24 d of age.

Choline chloride in basal diet of this study was 1025 mg/kg, which was 60 and 78% of the recommended level suggested by NRC (1994) and ROSS 308 Nutrition Specifications (2014), respectively. Although broiler chicken requirements for choline during 1-21, 22-42, and 43-56 d of age are about 1300, 1000, and 750 mg/kg diet based on NRC (1994), respectively, commercial broiler diets normally contain substantially higher choline concentrations. Pesti *et al.*, (1980) estimated choline requirement to be about 2130 mg/kg diet by the broken-line linear model for broiler chicks. They mentioned that choline/methionine levels depend on the price and availability of methionine supplements as well as the price of choline (Pesti *et al.*, 1980).

This study showed that ECC reduced moisture absorption by 550 g/kg but hydrophobic wall around the ECC did not reduce choline availability in GIT compared to CC group. This could be attributed to either easy penetration of enzymes into the wall pores of ECC or increased secretion of digestive enzymes (Noy and Sklan, 1995).

Conclusion

In conclusion, ECC and CC supplements had similar effect on growth performance and serum lipid indices, indicating no physiological consequences due to physico-chemical properties of choline supplements. However, the novel ECC product with hydrophobic characteristics in practical condition could be used to prevent caking agglomeration, and thus improves feed quality indices.

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