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Effects of partial replacement of conventional limestone with dietary micro-calcium carbonate on performance, egg quality, hematology, and calcium metabolism of laying hens during peak production

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Abstract: The present study was conducted to evaluate the effects of limestone replacement with dietary micro-calcium carbonate (MCC) on performance, egg quality, and serum Ca metabolism in peak-phase laying hens. A total of 120 laying hens (30 wk of age) were assigned to 1 control or 4 treatment groups for 8 weeks. Half of the total limestone was replaced by calcite in different particle sizes in the treatment groups (1, 20, 40, and 100 µm) and by fine limestone (~2 mm) in control. During the experiment, egg production and mass decreased ($p < 0.05$); however, eggshell thickness increased in hens fed with calcite groups compared to the control fed birds. There was a contrast between control vs. treatment, and a negative linear trend ($p = 0.11$) with the particle sizes for feed conversion ratio (FCR) was recorded. The different particle size of calcite had no effect on feed consumption, egg quality parameters, and hematology parameters of serum. However, the serum calcium ($p = 0.01$) and vitamin D ($p < 0.0001$) levels were affected by calcite, serum parathyroid hormone, calcitonin, phosphorus levels, and Ca:P ratio were not affected. The effects of dietary calcite sources may differ from conventional or nano-sized calcium carbonate sources in laying hens, especially for Vit D metabolism.

Key words: Calcite, micronized calcium, nano minerals, particle size, vitamin D

1. Introduction

Calcium (Ca) is an essential mineral for numerous enzymatic and biochemical processes in the metabolism of laying hens. Due to shell calcification, Ca requirements of laying hens are very high in the production cycle, particularly during the peak of egg production period from 25 to 39 weeks of age. During the peak stage of the laying period, Ca homeostasis is a big challenge for metabolism since shell formation requires several times as much calcium as the amount present in the extracellular pool [1]. Therefore, major sources of Ca minerals are intestinal absorption of dietary Ca and mobilization of bone Ca. The physical form of dietary Ca may influence its absorption in laying hens. The particle size of the Ca probably has a more significant effect on eggshell quality than the Ca level in layer diet [2–4].

Limestone consists of calcite and aragonite, which are different forms of calcium carbonate ($CaCO_3$), and it is commonly used in diets of laying hens as a Ca source [5]. The particle size of limestone more than 1 mm can improve the available Ca amount in the gastrointestinal tract (GIT) since retention times of larger limestone particles prolong in gizzard [6]. An increased amount of available Ca in the GIT can protect from metabolic disorders caused by excessive bone mobilization of Ca and impaired shell calcification followed by poor eggshell quality. Furthermore, dietary Ca affects the pericecal utilization of phosphorus (P) [7]. Some evidence suggests that providing large particle sizes varying from 25% to 80% of total Ca sources of diet may improve eggshell quality [2]. Although different combinations of larger particle size limestone and fine particle limestone have positive effects on the performance of laying hens, as previously mentioned by Koreleski and Swiatkiewicz [8], it was reported that laying performance and feed conversion ratio (FCR) were lower for layers provided with entirely large particle size of dietary Ca source than the layers fed a combination of large and fine particle size of dietary Ca sources [8].

Although there is minimal data available about the effects of feeding duration of dietary Ca on laying hens,

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Al-Batshan et al. [9] reported a significant linear decline in intestinal Ca uptake, bone ash content, and eggshell quality of laying hens from the peak production (35–37 weeks of age) to end of the production cycle. Therefore, Safaa et al. [10] observed that the dietary Ca requirement of laying hens has a progressively increasing trend from peak production to the late phase of the production instead of reported by NRC [11]. Therefore, the researchers suggest that increasing the Ca content of the diet may be a good strategy to avoid the disadvantages mentioned above on the eggshell quality and production over time. Swiatkiewicz et al. [3] observed the effects of increasing dietary Ca concentration (from 3.20% to 4.20%) on laying hens during peak production. Even though they reported no significant differences in laying performance and eggshell quality, stiffness and breaking strength of bones improved linearly with dietary Ca content. According to the previous reports, we hypothesized that the same improvement might be possible with an increased dietary Ca level and enhanced Ca availability during the peak production cycle of laying hens.

Over the last decade, due to environmental concerns, many studies have focused on strategies that can help avoid excessive mineral usage and adjust the optimal supplementation of dietary minerals in animal diets. Increasing the bioavailability of dietary minerals via nanotechnology applications may reduce the amount of minerals used in the diets. In animal nutrition, nanotechnology has been a hot topic for the last two decades [12]. Evidence suggested that nano- and micro-minerals could give rise to better interaction with other biological substances and higher utilization in animals than the conventional counterparts due to the larger surface area and unique chemical properties of nano- and micro-minerals [12]. Ganjigohari et al. [4] suggested that a level of 0.2% to 2% nano-$CaCO_3$ can be used instead of 4% conventional $CaCO_3$ in laying hens' diets to some dose-dependent positive effects (e.g., thicker eggshell) of nano Ca carbonate supplementation on laying hens. However, the researchers suggest a mild supplementation level due to adverse or lacking effects on performance parameters at extreme levels of nano-$CaCO_3$ in the same study. Nano minerals are characterized by a particle size of 1 to 1100 nm [13]. Therefore, the calcite products were expressed as 'micro-$CaCO_3$' (calcite) in the present study because they had 1, 20, 40, and 100 μm particle sizes. Although there have been some comparative reports about the effects of nano- and micro-size Ca sources versus conventional size Ca sources in diets of laying hens, there has been no comparative study between different particle sizes of the same micro-calcite sources in laying hen diets, to the best of our knowledge.

Therefore, the objective of this study was to investigate the effects of dietary calcite instead of conventional $CaCO_3$ sources on performance, egg quality, and hormonal Ca metabolism in laying hens. Our secondary objective was to provide comparative data with the different calcite particle sizes in laying hens.

2. Materials and methods

All experimental procedures used in this experiment were approved by the Animal Ethics Committee of the Afyon Kocatepe University (Case No.49533702/128).

2.1. Characterization of calcite product and particle size distribution

The calcite was provided by Nigtas Calcite Trade & Industry Co., Ltd. (Nigde, Turkey; 99.99% micronized calcite). Each calcite was a pulverized white powder with particle sizes of 1 μm, 20 μm, 40 μm, and 100 μm, and the particle sizes were validated by the company. The calcite was characterized by a field emission scanning electron microscope (Zeiss-SEM, Carl Zeiss AG, Oberkochen, Germany) (Figure 1). The particle size distribution of each calcite was determined using a particle analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) within the water as a dispersant (Figure 2).

2.2. Birds and experimental design

The study was conducted at Afyon Kocatepe University Animal Teaching & Research Center, Afyonkarahisar, Turkey. A total of 120 Babcock white laying hens, 30 weeks of age, with an initial body weight of 1586.9 ± 12.1 g were used in the study. The birds were randomly assigned to one of 5 groups having one control and four treatment groups (n = 24) with a completely randomized block design. As an experimental unit, each group cage contains 24 birds with four subgroups, and further, each subgroup contains six hens. Animals were housed in battery cages (width = 48 cm; depth = 45 cm; height = 45 cm) (2 birds/cage, at a density of 1080 cm^2/hen, 3 cages with same feeder in the same line, 4 floors per experimental group). The temperature of the room was recorded 24 h/day throughout the study. Sixteen hours (10 h sunlight plus 6 h artificial) of light (20 lux fluorescent light) and 8 h of dark were provided to the laying hens along with ad libitum feed and free access water.

The basal diet was formulated and provided for all experimental groups, as shown in Table 1 [11]. The control diet had no supplementation of calcite and had fine + coarse (50 :50) particle size of conventional limestone (particle sizes 0.20–0.50 and 2–3 mm, respectively). The treatment groups' diets were supplemented with calcite at a different particle size of 1 μm, 20 μm, 40 μm, and 100 μm for 8 weeks. In calcite groups (1 μm, 20 μm, 40 μm, and 100 μm), 50% of the total dietary Ca source was provided from coarse limestone, and 50% of total limestone was

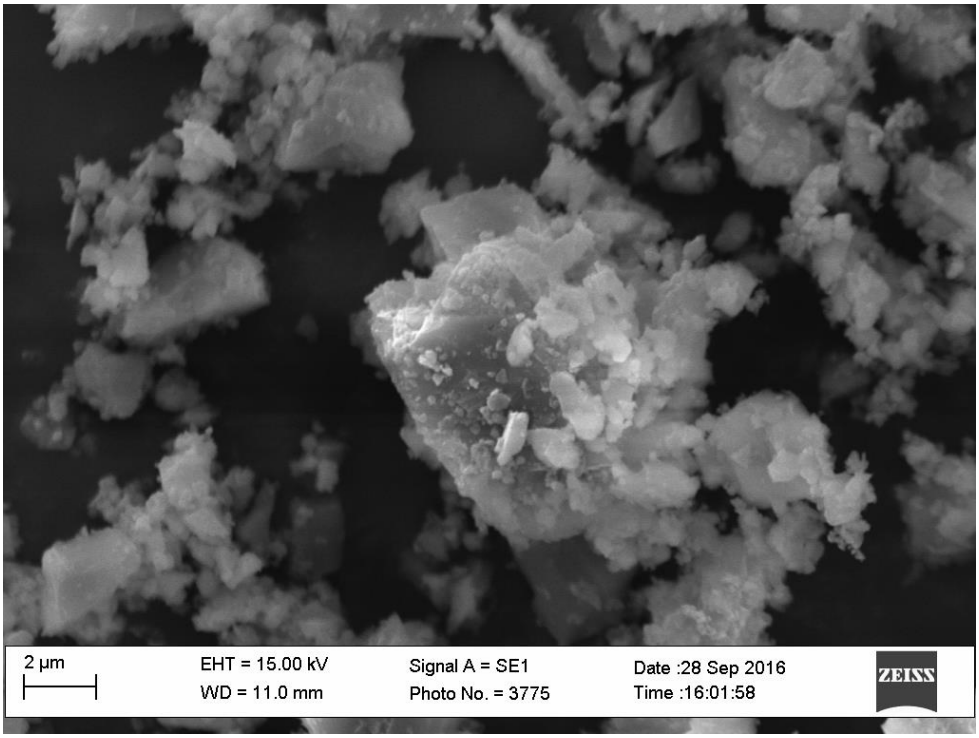

Figure 1. Morphology of micro-calcium carbonate particles (1 µm) in the scanning electron microscope.

provided from the relevant particle size of calcite to avoid metabolic disorders caused by the finest particle size of calcium sources for the whole diet [2].

2.3. Data collection, calculations, and egg trait analyses

The hens used in this study were weighed at the beginning and at the end of the study to determine their live weights. The mortality rate was zero during the study. Hen-day egg production was recorded daily (HDEP), while the hens' feed consumption was recorded weekly. Each feeder's total mixed feed was initially weighted due to three subgroup cages with the same feeders for each line. Then the residuals were weighted for each feeder. Egg mass (EM) was calculated as follows [14]:

EM (g)=HDEP (%)×Egg weight (g)

FCR values were calculated as follows [14]:

FCR=Feed intake (g)÷Eggmass (g)

Eggs were delivered to the laboratory at the end of the 4th week and at the end of the 8th week, with three egg samples from each subgroup to determine the egg quality parameters such as egg weight, breaking strength, and eggshell thickness. The eggs were kept for 24 h at room temperature before the egg trait analyses. Eggshell breaking strength (EBS) was measured by using ORKA Egg Force Reader, EF 0468-2011, and the Haugh Unit was calculated as follows:

Haugh Unit=100×log (h-1.7w$^{0.37}$+7.6)

where *h* is albumen height in mm and *w* is egg weight in grams.

Eggshell thickness was measured by a digital micrometer on four different parts (blunt end, sharp end, and two sides of equator of eggs) of each eggshell for each egg. Egg yolk color was determined using Roche Improved Yolk Color Fan and comparing the colors of yolks with 15 bands of the color spectrum.

2.4. Blood sampling and analyses

At the end of the trial, 3 hens were randomly selected from each subgroup, and blood was collected from the heart. The blood samples were transferred into two separate tubes (vacutainer tubes without anticoagulant or with Ethylenediaminetetraacetic acid-EDTA). All samples arrived immediately at the laboratory under a cold chain. The samples in EDTA tubes were analyzed for hematological parameters (Total leukocyte count, TLC; Lymphocyte count, LC; Neutrophil count, NC; Monocyte count, MC; Red blood cell count, RBC; Hemoglobin, He; Hematocrit, Ht; Mean corpuscular volume, MCV; Mean corpuscular hemoglobin, MCH; Mean corpuscular hemoglobin concentration, MCHC; Platelet, PLT; Mean platelet volume, MPV) by a total blood analyzer (BC 2800 Vet, Mindray Medical International Ltd., Shenzhen, China). For serum biochemical analyses, the samples in vacutainer tubes were centrifuged at 5000 rpm for 10 min. The supernatants were transferred to eppendorf tubes and stored at –20 °C till biochemical analyses. Serum glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate aminotransferase

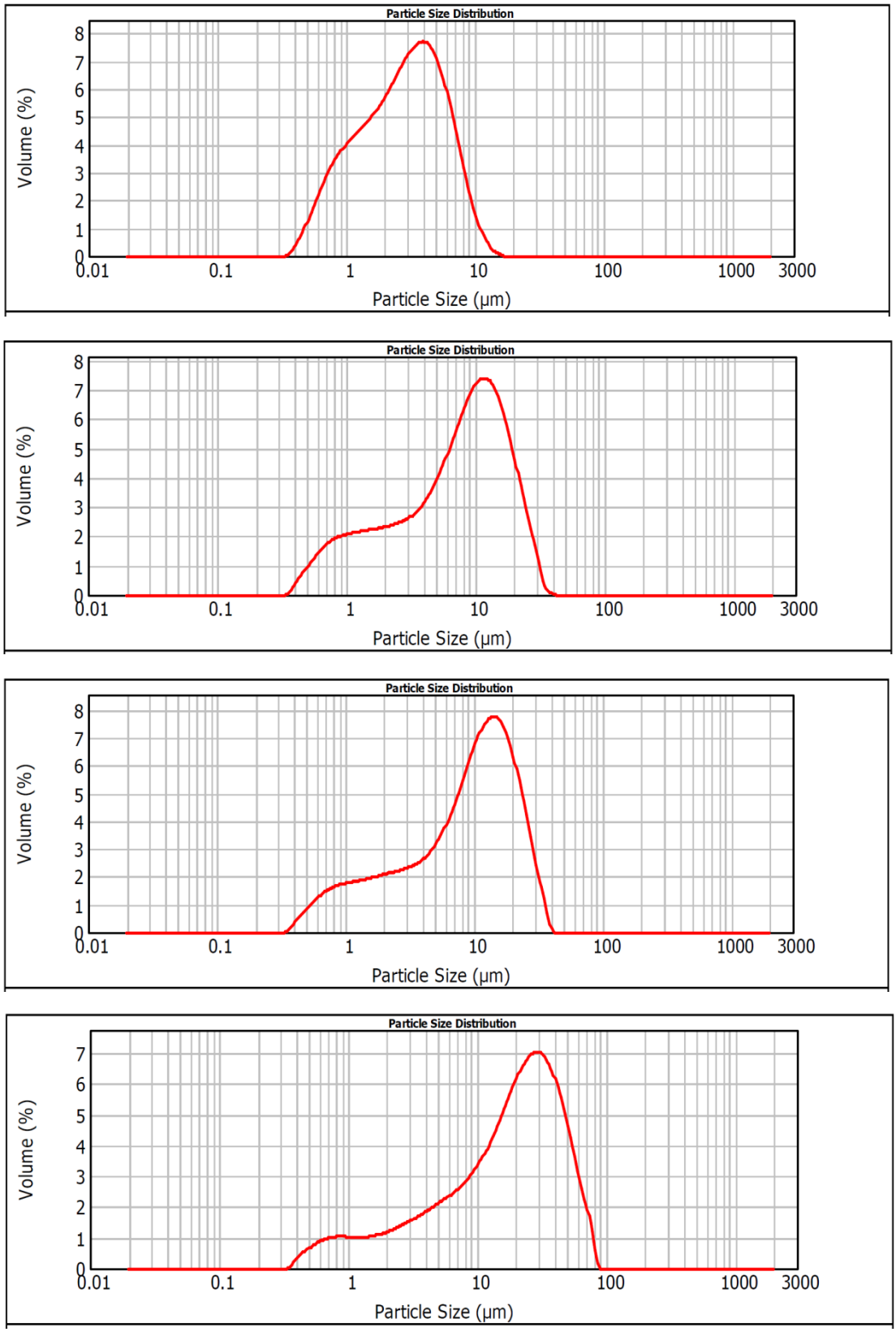

Figure 2. The particle size distribution of micro-calcium carbonate particles in water. **A:** 1 µm calcite; **B:** 20 µm calcite; **C:** 40 µm calcite; **D:** 100 µm calcite.

Table 1. Composition of the experimental diets.

Feed ingredients, % as feed	Control diet	Calcite diet
Maize, grain	54.90	54.90
Vegetable oil	0.34	0.34
Sunflower meal, 32% CP	16.93	16.93
Full-fat soybean meal, 34% CP	10.0	10.0
Soybean Meal, 44% CP	7.39	7.39
Limestone[1]	7.88	3.94
Calcite[2]	-	3.94
Dicalcium phosphate	1.73	1.73
Salt	0.38	0.38
Vitamin and mineral premix[3]	0.25	0.25
L-lysine hydrochloride	0.10	0.10
DL-methionine	0.10	0.10
Total	100.00	100.00
Chemical composition, calculated		
Dry matter (DM), %	87.50	87.50
Metabolizable energy, kcal/kg DM[4]	2750	2750
Crude protein, % DM	16.97	16.97
Ether extract, % DM	4.06	4.06
Crude fiber, % DM	5.59	5.59
Ash	11.47	11.47
Calcium, % DM[4]	3.71	3.71
Available phosphorus, % DM[4]	0.38	0.38
Sodium, % DM[4]	0.20	0.20
Lysine, % DM[4]	0.83	0.83
Methionine+Cysteine, % DM[4]	0.71	0.71
Threonine, % DM[4]	0.61	0.61
Tryptophan, % DM[4]	0.20	0.20
Linoleic acid, % DM[4]	2.36	2.36

[1)] Fine+coarse particle (50:50) as a conventional source.
[2)] 1 µm particle size in group *Cal1*; 20 µm particle size in group *Cal20*; 40 µm particle size in group *Cal40*; 100 µm particle size in group *Cal100*.
[3)] Provided per kg of diet: Vitamin A: 12000 IU; Vitamin D_3: 3000 IU; Vitamin E: 35000 IU; Vitamin K_3: 3500 IU; Vitamin B_1: 2750 IU; Vitamin B_2: 5500 IU; Nicotinamid: 30000 IU; Ca-D-Panthotenate: 10000 IU; Vitamin B_6: 4000 IU; Vitamin B_{12}: 15 IU; Folic acid: 1000 IU; D-Biotin: 50 IU; Cholin chloride: 150000 IU; Manganese: 80 mg; Iron: 60 mg; Zinc: 60 mg; Copper: 5 mg; Iodine: 2 mg; Cobalt: 500 mg; Selenium: 150 mg; Antioxidant: 15 mg.
[4)] Calculated.

(AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), vitamin D (vitD), parathormone (PTH), calcitonin, total protein, total phosphorus (tP), and total calcium (tCa) concentrations were determined by an automated ELISA analyzer (Elisys Uno, Human mbH, Wiesbaden, Germany) in a commercial laboratory.

2.5. Statistical analysis

The data were analyzed in a randomized block design with the cage as the experimental unit. The data were evaluated using PROC GLIMMIX of SAS (SAS Institute Inc, 2009). The micro-calcium treatment (control, Cal1, Cal20, Cal40 or Cal100) was fixed effects and cage was the random

Table 2. Effects of different dietary micro-calcium particle sizes on performance parameters of laying hens for 8 weeks[1].

Items	Feed consumption, g/hen/day	HDEP[2], %	FCR[3]	Egg weight, g/day	Egg mass, g/day
Control	97.83	82.80[a]	1.19[a]	60.42	50.05[a]
Cal1	92.19	65.37[b]	1.47[b]	58.93	38.54[b]
Cal20	95.49	76.52[a]	1.26[a]	58.35	44.66[c]
Cal40	93.41	68.53[b]	1.39[a]	59.53	40.90[bc]
Cal100	99.82	70.60[b]	1.45[a]	59.94	42.27[bc]
SEM[4]	3.49	2.75	0.07	0.98	1.75
P-values	0.54	0.0024	0.05	0.61	0.0026
Contrast[5]					
Cont *vs.* Calcites	0.51	<.0001	0.02	0.28	<.0001
Linear	0.30	0.18	0.11	0.69	0.24
Quadratic	0.43	0.42	0.85	0.49	0.31

[1)] Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n = 24). Control = basal diet supplemented with fine+coarse particle limestone (50:50); Cal1 = basal diet supplemented with 1 µm micronized calcite+coarse particle limestone (50:50); Cal20 = basal diet supplemented with 20 µm micronized calcite+coarse particle limestone (50:50); Cal40 = basal diet supplemented with 40 µm micronized calcite+coarse particle limestone (50:50); Cal 100 = basal diet supplemented with 100 µm micronized calcite+coarse particle limestone (50:50).
[2)] Hen day egg production.
[3)] Feed conversion ration
[4)] Standard error of the mean
[5)] Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS.
[a,b,c] Values with different superscripts in the same column are significantly different ($p \leq 0.05$) and for tendency declared at $0.05 < p < 0.15$.

effect in the model. Studentized residuals were determined with all fixed effects and interactions. The results with studentized residue <–4 or >4 were considered outliers and removed from the model. Degrees of freedom were calculated using either Kenward–Roger or Satterthwaite approximation [15]. Multiple comparisons with a Tukey–Kramer adjustment were performed.

Contrasts of control vs. treatment diets, linear and quadratic contrasts were used to evaluate responses to the particle size of dietary calcium at control, 1 µm, 20 µm, 40 µm, 100 µm. Due to the unequally spaced particle sizes, coefficients of contrasts were calculated with PROC IML of SAS [16]. All data are reported as least square means ± pooled SEM in tables. The significance level was set at $p \leq 0.05$ for all data and was assumed at $0.05 < p < 0.15$ for tendency.

3. Results

The particle size distribution of each type of calcite was determined in water as 1 µm, 20 µm, 40 µm, and 100 µm (Figure 2).

3.1. Productive performance and egg quality

The performance parameters were shown in Table 2 and Figure 3. The HDEP and egg mass values of calcite groups were lower than the control birds ($p = 0.0024$ and $p = 0.0026$, respectively) except for the HDEP of Cal20 birds. Unlike others, the HDEP of Cal20 was similar to control. The feed conversion ratio had a main treatment effect ($p = 0.05$) in the Cal1 group compared with control birds. However, FCR had a contrast between control vs. treatment groups. The FCR showed a negative trend ($p = 0.11$) with the calcite particle size. Even though feed consumption was significantly different between the groups at week 8, it was not enough for a main treatment effect. Moreover, this case was validated by no observed contrast between control vs. treatment for feed consumption. There was no treatment effect for egg weight in the present study.

Egg trait values were shown in Table 3. Although calcite had no effect on EBS and egg yolk color in the study, there was a treatment effect on eggshell thickness ($p = 0.04$). The shell thickness of the Cal 40 fed hens was greater than the control birds (0.386 vs. 0.313 mm). There

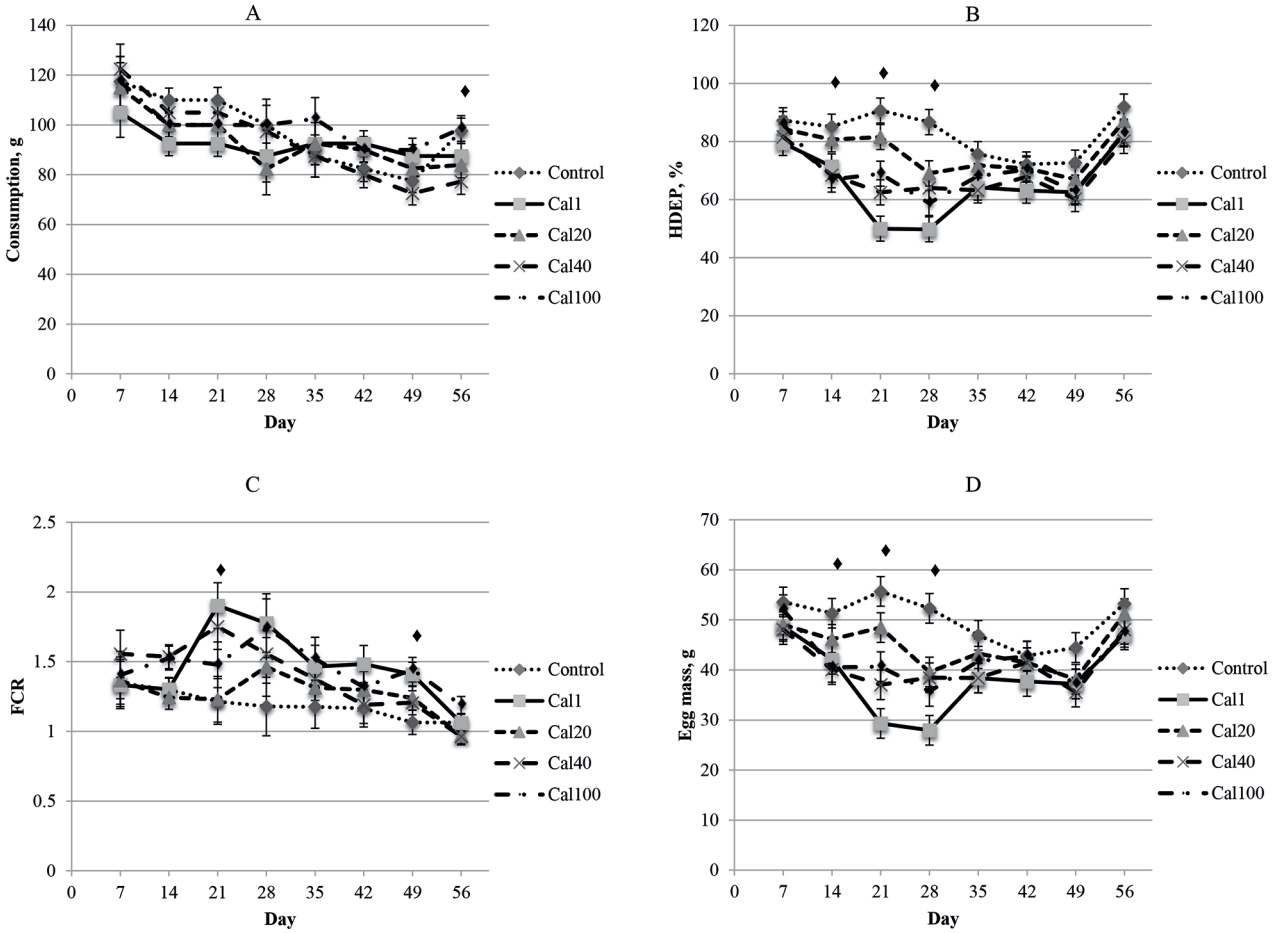

Figure 3. Effects of different dietary micro-calcium particle sizes on feed consumption (**A**), hen-day egg production (**B**), FCR (**C**), and egg mass (**D**) of laying hens for 8 weeks (Control, a basal diet supplemented with fine+coarse particle limestone; Cal1, a basal diet supplemented with 1 µm micronized calcite+coarse particle limestone; Cal20, a basal diet supplemented with 20 µm micronized calcite+coarse particle limestone; Cal40, a basal diet supplemented with 40 µm micronized calcite+coarse particle limestone; Cal100, a basal diet supplemented with 100 µm micronized calcite+coarse particle limestone. The sign (♦) represents significant differences between the groups at the time point. The main effects and contrasts are shown in Table 2.

was a significant contrast between control vs. treatment groups; however, there was no trend response to particle size ($p = 0.12$, $p = 0.35$, and $p = 0.01$, linear and quadratic, respectively). Interestingly, there was a contrast between control vs. calcite groups ($p = 0.13$); however, the Haugh unit had no main treatment effect.

3.2. Blood parameters

Serum biochemical parameters were shown in Table 4. The serum glucose, HDL, LDL, AST, ALT, total protein, and total cholesterol levels were not affected by calcite particle size. Serum GGT levels had the main treatment effect ($p = 0.05$). The higher serum GGT levels in control birds had a control vs. treatment contrast and a positive response to particle size ($p = 0.02$ and $p = 0.09$ (quadratic), respectively). Unexpectedly, calcite had no effect on serum PTH and calcitonin levels of laying hens. However, serum tCa and VitD levels had strong treatment effects ($p = 0.01$ and $p < 0.0001$, respectively). Serum tCa levels of Cal 20 group hens were higher than both Cal 1 and Cal 100 birds. Even though all treatment groups' serum tCa levels were similar to the control group, the highest serum concentration of tCa was in the Cal20 group. Also, serum VitD levels of the control layers were higher than all calcite groups, and it had a strong contrast between control vs. calcite groups. Serum tP levels were not affected by different dietary calcite particle size. The Ca:P ratio was shown to have no pooled treatment effect in the whole model or contrast between control vs. treatments. Dietary calcite did not affect any whole blood hematology parameters, as shown in Table 5, except trend on MCH and He ($P = 0.12$ for both, linear and quadratic, respectively).

Table 3. Effects of different dietary micro-calcium particle sizes on egg traits of laying hens for 8 weeks[1].

Item	EBS[2], kg cm^{-2}	Haugh unit	Egg yolk color	Eggshell thickness, mm
Control	41.16	75.10	6.38	0.313[b]
Cal1	39.14	69.53	6.00	0.330[ab]
Cal20	43.39	72.05	5.54	0.353[ab]
Cal40	38.42	71.40	6.38	0.386[a]
Cal100	43.00	71.85	6.04	0.341[ab]
SEM[3]	1.89	2.19	0.08	0.011
P-values	0.51	0.52	0.28	0.04
Contrasts[4]				
Cont *vs.* Calcites	0.95	0.13	0.26	0.12
Linear	0.49	0.84	0.98	0.35
Quadratic	0.64	0.75	0.87	0.01

[1)] Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n = 24). Control = basal diet supplemented with fine+coarse particle limestone (50:50); Cal1 = basal diet supplemented with 1 µm micronized calcite+coarse particle limestone (50:50); Cal20 = basal diet supplemented with 20 µm micronized calcite+coarse particle limestone (50:50); Cal40 = basal diet supplemented with 40 µm micronized calcite+coarse particle limestone (50:50); Cal100 = basal diet supplemented with 100 µm micronized calcite+coarse particle limestone (50:50).

[2)] Eggshell breaking strength.

[3)] Standard error of the mean.

[4)] Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS.

[a,b,c] Values with different superscripts in the same column are significantly different ($p \leq 0.05$) and for tendency declared at $0.05<p<0.15$.

4. Discussion

4.1. Productive performance and egg quality

In the present study, dietary micro-calcium sources did not affect feed consumption. Simultaneously, the egg production was lower in birds fed with micro-calcium than the control birds fed with conventional calcium sources ($p < 0.05$). Interestingly, FCR had a negative linear response to calcite particle size with a contrast between control vs. treatment groups. In the present study, the lower egg production recorded in calcite groups compared to control group may be the cause of observed contrast (control vs. micro-calcium groups) in FCR values calculated based on egg production. The results of the previous reports suggested that the particle size of dietary Ca source does not affect performance and egg trait in laying hens and broiler breeders [17–19]. Even Molnar et al. [20] compared the effects of limestone particle size in a conventional or splitting feeding system. There has been a limited number of reports about dietary nano- and micro-calcium effects on the performance and egg quality of laying hens. Ganjigohari et al. [4] reported that replacing about 30% of total conventional calcium carbonate with nano-calcium carbonate (15–40 nm) in diet had no significant effect FCR in laying hens in contrast with the present study. Also, feed consumption data is not available in the research mentioned above. The present study contrasts with Cufadar et al. [18] and Ganjigohari et al. [4], who reported no effect of particle size of dietary calcium source on egg production in laying hens. However, Cufadar et al. [18] focused on conventional particle size (2000 µm) of dietary calcium sources, while the present study evaluated micro-sized calcium sources (1–100 µm). Similarly, Ganjigohari et al. [4] assessed the effects of nano-sized calcium sources (0.015–0.040 µm) compared with the present study. Kim et al. [21] reported significant differences in cellular uptake potentials, biokinetics, and molecular behaviors among the dietary $CaCO_3$ sources with conventional, micro- and nano-particle sizes (discussed in detail in the next section).

The differences between the egg production and FCR values of this study and the above mentioned studies' values may be due to very different particle sizes of the studies' calcium sources. The whole model evaluation, the HDEP, egg mass, and FCR were significantly different in the treatment groups at some individual sampling point until 28 days of the study when weekly evaluations are taken into account. However, the calcite particle size had only time×treatment interaction for HDEP and egg mass, not FCR. On a weekly basis, it makes sense with the lack of interaction in feed consumption included in FCR calculation.

The present study results showed that eggshell thickness was greater in hens fed with micro calcium than the birds fed with conventional Ca sources. However, micro calcium sources did not affect eggshell breaking strength. Thicker eggshells in hens fed with micro-calcium sources compared with the birds fed traditional Ca source, together with similar plasma Ca levels, suggest that micro calcium may have caused more Ca accumulation in the shell. Although there was no data about Ca content of eggshell in our study as a limitation of the research, increased shell thickness without changes on egg weight indicates more Ca content in the eggshell due to around 95% Ca content of dry matter of eggshell [2]. The data on the effects of dietary micro calcium sources on eggshell thickness have been limited. However, Ganjigohari et al. [4] reported that a similar amount of dietary nano-sized calcium resulted in a significantly thicker eggshell than the conventional limestone size in laying hens, in agreement with the results of the present study. Furthermore, Cufadar

Table 4. Effects of different dietary micro-calcium particle sizes on serum biochemical parameters of laying hens for 8 weeks[1].

Item	Glucose, mg/dL	Cholesterol, mg/dL	HDL[2], mg/dL	LDL[3], mg/dL	AST[4], U/L	ALT[5], U/L	GGT[6], U/L	VitD[7], ng/mL	PTH[8], pg/mL	Calcitonin, pg/mL	Total Protein, g/dL	Phosphorus, mg/dL	Calcium, mg/dL	Ca:P
Control	220.04	84.50	20.62	35.70	187.94	2.08	28.10	10.02[a]	2.55	3.36	4.81	4.70	18.69[ab]	4.03
Cal1	202.11	92.80	18.88	46.70	211.01	1.89	17.33	2.08[b]	3.01	5.22	5.14	3.93	13.51[b]	4.04
Cal20	216.58	99.60	17.62	41.10	203.35	5.92	19.44	3.49[b]	2.54	6.32	5.52	5.23	22.89[a]	4.55
Cal40	195.68	122.60	21.02	59.50	300.98	2.13	18.80	2.03[b]	1.45	5.05	5.71	3.46	17.55[ab]	5.02
Cal100	217.70	97.00	19.57	44.89	218.38	4.78	24.89	2.14[b]	2.70	2.75	5.47	4.22	14.48[b]	3.43
SEM[9]	13.58	16.85	2.07	10.33	44.74	1.55	2.79	0.63	0.83	1.67	0.43	0.57	1.87	0.46
p-values	0.64	0.59	0.78	0.57	0.45	0.27	0.05	<.0001	0.75	0.58	0.61	0.28	0.01	0.17
Contrasts[10]														
Cont *vs.* Calcites	0.44	0.33	0.57	0.29	0.38	0.37	0.02	<.0001	0.90	0.46	0.18	0.46	0.45	0.66
Linear	0.82	0.62	0.89	0.65	0.59	0.28	0.53	<.0001	0.86	0.40	0.36	0.61	0.24	0.31
Quadratic	0.40	0.14	0.93	0.26	0.15	0.76	0.09	0.00	0.26	0.31	0.21	0.64	0.04	0.02

[1)] Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n = 24). Control = basal diet supplemented with fine+coarse particle limestone (50:50); Cal1 = basal diet supplemented with 1 µm micronized calcite+coarse particle limestone (50:50); Cal20 = basal diet supplemented with 20 µm micronized calcite+coarse particle limestone (50:50); Cal40 = basal diet supplemented with 40 µm micronized calcite+coarse particle limestone (50:50); Cal100 = basal diet supplemented with 100 µm micronized calcite+coarse particle limestone (50:50).
[2)] High-density lipoprotein.
[3)] Low-density lipoprotein.
[4)] Aspartate aminotransferase.
[5)] Alanine aminotransferase.
[6)] Gamma-glutamyl transpeptidase.
[7)] Vitamin D, 25-Hydroxy.
[8)] Parathormone.
[9)] Standard error of mean.
[10)] Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS.
[a,b,c] Values with different superscripts in the same column are significantly different ($p \leq 0.05$) and for tendency declared at $0.05<p<0.15$.

et al. [18] indicated that the large particle size of limestone resulted in the best egg quality when dietary Ca is low. However, the researchers suggest the finest limestone particle size for the best external egg quality when dietary Ca concentration is enough, even though they focused on greater particle size than micro-sized calcium used in the present study.

4.2. Blood parameters

Although calcite did not affect serum PTH, calcitonin, tP levels, and serum Ca:P ratio of laying hens, serum tCa, and VitD levels had strong treatment effects in the present study. Serum VitD levels of the control birds were higher than all calcite groups, and it had a strong contrast between control vs. calcite groups. Serum tP levels was not affected by dietary calcite. The Ca:P ratio was shown that there was no pooled treatment effect in the whole model or contrast between control *vs.* treatments. Ajakaiye et al. [22] reported that there was no significant differences between in vitro solubilities of two different particle sizes of the same calcium carbonate sources (> 500 µm vs. < 500 µm). However, Safaa et al. [10] observed that in vitro solubility of limestone with 337 µm particle size was numerically higher than the limestone with 3360 µm particle size (57% vs. 33%, respectively) although Ca composition were similar in both limestone sources. Moreover, Zhang and Coon [23] concluded that calcium's in vitro solubility is negatively correlated with both in vivo solubility and gizzard retention of dietary Ca source in laying hens. However, the same researchers stated that a combination of in vivo solubility of dietary Ca sources for entire GIT and retention time of limestone in gizzard may not wholly represent Ca absorption due to the effects of kidney on Ca metabolism in the body [23]. Therefore, we focused on regulatory hormones of Ca metabolism and serum levels of tCa and tP in the present study. Kim et al. [21] reported that although plasma Ca concentration reached at the peak level significantly earlier in rats treated with a single oral dose of nano-$CaCO_3$ (0.1 µm) than the rats treated

Table 5. Effects of different dietary micro-calcium particle sizes on full blood counts of laying hens for 8 weeks[1].

Item	TLC[2] $\times10^9$/L	LC[3] $\times10^9$/L	NC[4] $\times10^9$/L	MC[5] $\times10^8$/L	RBC[6] $\times10^{12}$/L	He[7] g/dL	Ht[8] %	MCV[9] fL	MCH[10] pg	MCHC[11] g/dL	PLT[12] 10^{10}/L	MPV[13] fL
Control	1.98	1.71	0.74	0.428	2.61	10.97	34.67	108.99	30.20	31.30	26.51	6.56
Cal1	2.40	1.79	0.69	0.432	2.75	10.74	33.94	108.02	31.39	31.48	26.78	6.53
Cal20	2.43	1.75	0.73	0.427	2.66	10.56	34.52	107.24	30.27	30.73	26.37	6.48
Cal40	2.06	1.77	0.67	0.448	2.67	10.40	34.53	109.01	31.05	31.44	26.61	6.59
Cal100	2.51	1.73	0.76	0.429	2.69	10.74	34.24	108.93	29.65	31.24	27.03	6.56
SEM[14]	0.23	0.04	0.04	0.008	0.05	0.25	0.47	0.66	0.50	0.50	0.53	0.09
p-values	0.38	0.78	0.63	0.46	0.47	0.56	0.81	0.28	0.15	0.84	0.92	0.93
Contrasts[15]												
Cont *vs.* Calcites	0.16	0.34	0.59	0.58	0.17	0.20	0.49	0.37	0.50	0.89	0.75	0.85
Linear	0.35	0.83	0.58	0.87	0.77	0.71	0.88	0.40	0.12	0.97	0.51	0.75
Quadratic	0.63	0.66	0.37	0.20	0.80	0.12	0.62	0.71	0.40	0.78	0.64	0.98

[1)] Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n = 24). Control = basal diet supplemented with fine+coarse particle limestone (50:50); Cal1 = basal diet supplemented with 1 µm micronized calcite+coarse particle limestone (50:50); Cal20 = basal diet supplemented with 20 µm micronized calcite+coarse particle limestone (50:50); Cal40 = basal diet supplemented with 40 µm micronized calcite+coarse particle limestone (50:50); Cal 100 = basal diet supplemented with 100 µm micronized calcite+coarse particle limestone (50:50).
[2)] Total leukocyte count.
[3)] Lymphocyte count.
[4)] Neutrophil count.
[5)] Monocyte count.
[6)] Red blood cell count.
[7)] Hemoglobin.
[8)] Hematocrit.
[9)] Mean corpuscular volume.
[10)] Mean corpuscular hemoglobin.
[11)] Mean corpuscular hemoglobin concentration.
[12)] Platelet.
[13)] Mean platelet volume.
[14)] Standard error of the mean.
[15)] Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS.

with micro-$CaCO_3$ (2 µm), there were no differences between total plasma Ca concentration (AUC) and peak level of plasma Ca of micro- and nano-$CaCO_3$ after 6 h. The evidence suggested that the particle size of dietary $CaCO_3$ may not affect the absorption of Ca in GIT for the long term. The mentioned results of Kim et al. [21] were consistent with the present study, which had no effect of dietary micro-$CaCO_3$ compared with conventional dietary $CaCO_3$ on essential hormones of Ca metabolism such as PTH and calcitonin. These regulatory hormones may respond to the continuous daily intake of micro-$CaCO_3$ in the same manner as a conventional calcium source due to similar long-term absorption kinetics of both particle sizes. Moreover, the rapid plasma Ca turnover of laying hens (~1 min half-life) may explain the lack of long-term (8 weeks) treatment effects of dietary micro-calcium on serum tCa, tP, PTH, and calcitonin levels in our study. Swiatkiewicz et al. [3] observed no significant effect of partial replacement of coarse limestone with fine limestone on serum Ca and P levels of laying hens. Moreover, Manangi et al. [19] reported that no significant differences were observed between plasma tCa and plasma tP levels of broiler breeders fed different limestone particle sizes (185 *vs.* 3489 µm) after 24 h of oviposition in agreement with the present study.

Recent evidence in cellular Ca metabolism in the birds suggested that Ca transportation occurs in two pathways in the intestinal membrane and eggshell gland: transcellular pathway and paracellular pathway [1]. The transcellular pathway is an energy-dependent process, although the paracellular way uses a passive transport route with electrical potential differences. Some specific

membrane proteins should be involved in transcellular pathways, such as plasma membrane calcium-ATPases and calcidin, even though the paracellular route uses the way across tight junction proteins, which connect between epithelial cells and occur intestinal barrier. Therefore, the paracellular route is considered a lower-cost metabolic way than the transcellular route. The transcellular pathway is usually used when there is an insufficient concentration of Ca ions. Bar [1] concluded that paracellular transport is more critical in animals fed sufficient dietary Ca due to significant electrical potential differences between the intestinal environment and blood.

Furthermore, Powell et al. [24] suggested that although intestinal epithelium has a solid mucosal barrier with tight junctions, tiny particles can gain access to paracellular route under some conditions as dietary changes and productional stress factors. Bar [1] reported that Ca transportation in the eggshell gland is very similar to the intestinal transportation mechanism. The mucosal barrier and expressions of tight junctions in the eggshell gland can be disrupted in high-producing commercial laying hens [25]. This evidence suggests that micro-calcium may have more access to the mucosal barrier in the lower oviductal system of laying hens than the intestinal membrane. This hypothesis can be supported by greater eggshell thickness in treatment groups than the control birds, as discussed in the previous section.

In the present study, the sharp decrease of serum VitD levels in micro calcium groups but no change in serum PTH and calcitonin suggests that micro-calcium may be more effectively transported in the eggshell gland than the intestinal brush border membranes. The reason is that the complex relationship between vitD and Ca transportation is different in the eggshell gland of birds. However, the vitD dependency of intestinal Ca transportation of laying hens is similar to mammalians and other higher vertebrates [1]. For instance, Calbindin proteins, an essential membrane protein in Ca transport, act in a vitD-dependent manner in the intestinal membrane. However, the vitD is not necessary for the functions of Calbindin proteins in the eggshell gland. However, recent evidence suggests that paracellular transportation of Ca is still has a vitD (1,25(OH)2D3)-dependency [26].

In the present study, there was no detrimental effect of dietary micro-calcium on serum biochemical parameters, the activity of liver enzymes, and hematology, in agreement with the results of Huang et al. [13] and Kim et al. [21]. Huang et al. [13] observed no adverse effect, organ toxicity, or mortality of oral consumption of micro-calcium sources (3.7 μm) up to 2.3 g/kg of body weight in mice models. Moreover, Kim et al. [21] reported that micro-$CaCO_3$ (2 μm) treatment did not cause reactive oxygen species (ROS) and LDH production from human intestinal INT-407 cells after 24 h of incubation while nano-$CaCO_3$ (0.1 μm) gave rise to significantly higher ROS and LDH output from the cells than the counterpart. The mentioned research suggests that micro-calcium may be safer than nano-size calcium sources for enterocytes [21].

In conclusion, results from this study indicate that replacing 50% of dietary limestone with micro-sized calcite (1-100μm) did not affect most of the egg traits, performance, blood parameters, and Ca metabolism hormones of laying hens during the peak production, except creating adverse effects on egg production and a substantial impact on serum vitD levels. It is suggested that the biokinetic of micro calcium may be different from conventional size calcium sources in the eggshell gland rather than GIT. Further comparative studies on vitamin D-dependent-membrane proteins involved in Ca transportation in the eggshell gland may better help understand the effects of feeding laying hens with micro-calcium sources compared with conventional size calcium sources.

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