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Authors' contributions Please specify the authors' role using this form.	Conceptualization: Lee KW Data curation: Chang YQ, Park DH Formal analysis: Chang YQ, Park DH Methodology: Chang YQ, Park DH Software: Park DH Validation: Chang YQ, L S Investigation: Chang YQ, Kim DH, Lee WS Writing - original draft: Chang YQ Writing - review & editing: Chang YQ, Park DH, L S, Kim DH, Lee KW, Lee WS

Ethics approval and consent to participate	The experimental protocol used in this study was approved by the Animal Care and Use Committee (IACUC) of Konkuk University, Korea (approval number: KUB17074).
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6 **Abstracts**

7 This study investigated the biological effects of dietary conjugated linoleic acid (CLA) in
8 broiler chickens raised at different stocking densities. A total of 540 feather-sexed female broiler
9 chicks (Ross 308) were randomly assigned to one of 28 floor pens in a 2 × 2 factorial arrangement
10 with two stocking densities (800 cm² and 1,300 cm² per chick) and two levels of dietary CLA (0
11 and 10 g/kg). Each treatment consisted of seven replicates and the experiment lasted for five
12 weeks. Dietary CLA lowered the feed intake ($P = 0.038$) but improved the feed conversion ratio
13 ($P = 0.035$) in broiler chickens at day 21. Broilers raised under high vs. low stocking densities ate
14 and gained less during the finisher and whole phases. In contrast, high vs. low stocking densities
15 improved ($P < 0.001$) feed conversion ratio at the starter phase. None of the main factors (stocking
16 density and CLA) affected the rectal temperature and secretory immunoglobulin A content of the
17 ileal mucosa ($P > 0.05$). Tibial characteristics were negatively affected by stocking density at day
18 35. Dietary CLA increased the concentration of glutamic oxaloacetic transaminase ($P = 0.009$)
19 and total cholesterol ($P = 0.079$) in serum samples compared to the control group fed a CLA-free
20 diet. Antioxidant parameters, including superoxide dismutase and malondialdehyde, were not
21 altered ($P > 0.05$) by the stocking density or dietary CLA. Dietary CLA tended to increase
22 corticosterone levels in the serum samples of broiler chickens raised under a high stocking density,
23 exhibiting a partial interaction between the main factors ($P = 0.086$). Dietary CLA, but not
24 stocking density, altered the fatty acid composition of thigh meat ($P < 0.05$). In summary, dietary
25 CLA improved the fatty acid composition of broiler meat while maintaining normal growth
26 performance, and its efficacy was independent of stocking density.

27

28 **Keywords:** Broiler chickens, Conjugated linoleic acid, Fatty acid composition, Stocking density

INTRODUCTION

29

30

31 Broiler chickens are being farmed intensively at a high stocking density due to the global
32 demand for poultry meat [1]. However, intensive management has potential negative impacts on
33 the welfare and health of broiler chickens and can cause metabolic and leg disorders,
34 compromised immune responses, or increased susceptibility to disease [2]. In addition, it is
35 reported that high vs. low stocking density can alter fat metabolism via decreasing myristic acid
36 and increasing stearic acid and arachidonic acid, although it failed to affect the concentrations of
37 linoleic acid, linolenic acids, and total n-6 and n-3 polyunsaturated fatty acids in broiler breast
38 meat [3]. Various nutritional interventions have been used in the poultry industry, including the
39 administration of probiotics [4], r-aminobutyric acid [5], plant extracts [6,7], medium-chain fatty
40 acids [8], phosphorus-based products [9], and enzyme preparations [10,11].

41 Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic
42 acid containing a conjugated double-bond system, where each double bond can exist in either cis
43 or trans configuration (e.g., c9t11 and t10c12). CLA is derived from meat and dairy products of
44 ruminants and is naturally produced from polyunsaturated fatty acids in the rumen through
45 bacterial isomerization or biohydrogenation [12,13]. It is known that dietary CLA has multiple
46 beneficial health effects and provides anti-adipogenic, anti-carcinogenic, anti-atherosclerotic,
47 anti-diabetic, and anti-inflammatory functions [12]. In addition, dietary CLA regulates lipid
48 metabolism by suppressing fat tissue deposition and upregulating the saturated fatty acid
49 biosynthetic pathway [14,15]. It can also improve antioxidant homeostasis under oxidative stress,
50 regulate immune function [16,17] and maintain the gut health of broiler chickens [18]. As such,
51 dietary CLA is used to produce CLA-enriched poultry meat [19].

52 In contrast to the beneficial effects of dietary CLA, it has been found to have negative effects
53 on the growth performance of broilers, including the daily weight gain and the feed conversion
54 ratio [20,21,22]. Furthermore, it is currently unknown how dietary CLA influences growth
55 performance and whether it can be efficiently incorporated into chicken meat when broilers are

56 reared at high stocking densities. We therefore hypothesized that CLA elicits specific alterations
57 in the tissue fatty acid composition of broiler chickens and modulates key aspects of their
58 physiological stress response when reared under high-density conditions. We anticipated that this
59 functional modulation would occur independently of any significant effect on growth
60 performance. In addition to growth performance and fatty acid metabolism, stocking density is
61 known to comprehensively affect health and welfare of chickens [2] which urged us to measure
62 various markers including body temperature, tibia characteristics, and physiological indicators
63 (i.e., immune, stress, and antioxidant markers). The information obtained would be useful in
64 assessing the value of dietary CLA in broiler chickens and could lead to the formulation of
65 functional diets to be tested in further studies. As far as we know, this is the first report to study
66 the interaction between nutritional intervention and environmental stress (i.e., stocking density)
67 in broiler chickens.

68

MATERIALS AND METHODS

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71 **Ethics statement**

72 All experimental protocols and use of broilers in the trial were approved and certified by the
73 Institutional Animal Care and Use Committee of Konkuk University (KU17074).

74

75 **Experimental design**

76 A total of 540 d-old feather-sexed female broiler chicks (Ross 308) were randomly assigned to
77 one of four treatment groups with two stocking densities and fed diets with and without CLA.
78 Birds were housed in pens (1 × 2 m) at two stocking densities: high stocking density (HSD, 25
79 chicks per pen, 800 cm²/bird, 25.0 kg/m²) and low stocking density (LSD, 15 chicks per pen,
80 1,333 cm²/bird, 15.0 kg/m²), based on a final body weight of 2.0 kg. The CLA-enriched diet was
81 formulated by replacing 10 g/kg of soybean oil with CLA in corn-and soybean meal-based starter
82 and finisher control diets (Table 1). The CLA (Tonalin® TG 80, D-BASF, Düsseldorf, Germany)
83 used in this study contained 80% conjugated linoleic acid and a 50:50 ratio of the active
84 conjugated linoleic isomers (C18:2 c9,t11 and C18:2 t10,c12). The fatty acid compositions of the
85 experimental diets are shown in Table 2. Each treatment had seven replicates, and the experiment
86 lasted for 35 d. All pens contained a feeder (52-cm diameter) and nipples, and fresh rice husks
87 were used as the bedding material. The chicks were provided with free access to feed and water.
88 The temperature of the facility was set at 34°C followed by a gradual downward adjustment until
89 it reached at 24°C, and the humidity was maintained at approximately 60%. The light period was
90 23h of light and 1h of darkness. The body weight and feed intake of the chickens were monitored
91 weekly, and the mortality-adjusted feed conversion ratio was calculated.

92

93

94 **Sampling**

95 On 21 d and 35 d, one bird whose body weight was close to that of the pen's average was

96 randomly selected and euthanized by gradual fill of 10 – 30% of the chamber volume per minute
97 with carbon dioxide. Post-mortem examination confirmed that the carcasses sustained no physical
98 damage or traumatic injuries following asphyxiation. Immediately after euthanasia, blood
99 samples were collected via heart puncture. Serum samples were obtained by gentle centrifugation
100 at 200 g for 15 min and stored at –20°C before analysis. Immediately after blood sampling, the
101 meat from the entire right leg was removed to analyze its fatty acid composition and tibial
102 characteristics. A 5-cm-long mid-ileal segment was then sampled, cut longitudinally, and rinsed
103 with ice-cold saline. The ileal mucosa was collected by scraping the mucosal surface with a tissue
104 culture scraper, and it was thoroughly vortexed (C-VT Test Tube Vortex Mixer, Chang Shin
105 Scientific Co, Incheon, KOREA) with ice-cold saline, centrifuged (27,000 g × 20 min, 4°C), and
106 the supernatant obtained was stored at –20°C before assay. On 35 d, ileal segments and thigh meat
107 were only obtained to analyze ileal secretory immunoglobulin A (sIgA) and the fatty acid
108 composition of the thigh meat.

109

110 **Rectal temperature**

111 On 21 d and 35 d, three birds per pen were randomly selected to measure their rectal
112 temperatures, where a rectal thermometer was inserted to a depth of 3 cm.

113

114 **Tibia characteristics**

115 The tibia was obtained by manually removing the attached meat on 21 d and 35 d of the
116 experiment, and tibial length and thickness were recorded using standard digital calipers. The
117 tibia breaking strength was measured using an Instron (Model 3342, Instron Universal Testing
118 Machine, Instron Corp., Norwood, MA, USA) with a 50 kg load range and a crosshead speed of
119 50 mm/min, with the tibia supported on a 3.35 cm span. The results are expressed in this paper as
120 accumulated pressure values.

121

122 **Measurement of sIgA in ileal mucosa**

123 At 35 d, the sIgA levels in the ileal mucosa were analyzed using a commercially available

124 chicken-specific immunoglobulin A enzyme-linked immunosorbent assay kit (Bethyl
125 Laboratories Inc., Montgomery, TX, USA), as described previously [23]. The protein
126 concentrations in the supernatant samples were measured using a commercial BCA Assay kit
127 (Merck, Darmstadt, Germany). The sIgA concentrations are expressed in this paper as micrograms
128 of sIgA per milligram of total protein in the ileal mucosa samples.

129

130 **Serum biochemical, immunological, antioxidant and stress indicators**

131 On 35 d, serum samples were analyzed using an automatic blood chemical analyzer (Film DRI
132 CHEM 7000i, Fuji film, Tokyo, Japan) to determine total cholesterol, glucose, triglyceride,
133 glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and uric acid
134 levels. To measure the antioxidant parameters in the serum samples, the total antioxidant capacity
135 (TAC; QuantiChrom antioxidant assay kit, BioAssay Systems, Hayward, CA, USA),
136 malondialdehyde (MDA; OxiSelect thiobarbituric acid reactive substances assay kit, Cell Biolabs,
137 Inc, San Diego, CA, USA), and superoxide dismutase (SOD; EnzyChrom superoxide dismutase
138 assay kit, BioAssay Systems, Hayward, CA, USA) were assayed according to the manufacturer's
139 guidelines.

140 On 21 d and 35 d, nitric oxide in the serum samples was measured using a modified Griess
141 reagent (Sigma Aldrich, St. Louis, MO, USA), and the NO concentration was calculated from a
142 standard curve with sodium titrate. Corticosterone concentrations in the serum samples were
143 determined using a commercially available CORT ELISA kit (Enzo life science Inc, ADI-901-
144 097, Farmingdale, NY, USA), according to the manufacturer's instructions.

145

146 **Determination of fatty acid composition of thigh meat**

147 On 35 d, approximately 25 g of thigh meat was thawed and mixed with chloroform/methanol
148 (2:1, v/v) to extract lipids. Fatty acid methyl esters (FAME) were prepared using 0.66 N potassium
149 hydroxide in methanol and 14% methanolic boron trifluoride (Sigma Chemical Co., St. Louis,
150 Missouri, USA). The resulting FAMEs were analyzed by gas chromatography (HP 6890; Agilent
151 Technologies, CA, USA) using a 100 m × 0.32 mm i.d. capillary column (SP-2560, Supelco, Inc.,

152 Bellefonte, PA, USA). Peaks were identified by comparing equivalent chain lengths with a
153 reference standard (mix C4–C24 methyl esters; Sigma Aldrich, Inc., St. Louis, MO, USA) and a
154 CLA standard mix (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA; Sigma-Aldrich, Inc., St. Louis,
155 MO, USA). The fatty acid composition is expressed in this paper as a percentage of the sum of
156 the total identified peaks in each sample.

157

158 **Statistical analysis**

159 The pens were considered to be the experimental units. For variables measured in multiple
160 animals within a pen, the values were averaged to obtain a single pen-level value before analysis.
161 The data obtained in this study were analyzed using SAS (version 9.4; SAS Institute Inc., Cary,
162 NC, USA) with the General Linear Model (GLM) procedure for a 2×2 factorial design, with
163 stocking density and CLA and their interactions as the main factors. Differences were considered
164 to be statistically significant at $P < 0.05$. A probability level of $0.05 < P \leq 0.10$ was defined as
165 indicative of a statistical trend.

RESULTS

166

167

168 **Growth performance**

169 No significant interactions between the main factors (i.e., stocking density and CLA) on the
170 production indices were noted in any of the production phases (Table 3). High vs. low stocking
171 densities lowered ($P < 0.05$) feed intake in the finisher and whole phases. In the starter phase,
172 high-stocking-raised broilers ate 3.9% less ($P = 0.078$) than broilers raised under low-stocking
173 density. Dietary CLA lowered ($P = 0.038$) the feed intake in the starter phase, but the CLA-
174 mediated decrease in feed intake was not significant ($P > 0.05$) in the finisher or whole phase.
175 Body weight gain was lower ($P < 0.001$) in broilers raised at high vs. low stocking densities during
176 the finisher and whole phases. However, dietary CLA did not affect the body weight gain at any
177 age. Both stocking density and dietary CLA supplementation improved ($P < 0.05$) the feed
178 conversion ratio of broilers in the starter phase.

179

180 **Rectal temperature**

181 Rectal temperature ranged from 40.9°C to 41.3°C at 21 d and 35 d, and it was not significantly
182 affected ($P > 0.05$) by stocking density and dietary CLA. No significant interaction effect of
183 stocking density and CLA on rectal temperature was observed ($P > 0.05$) at any age (Table 4).

184

185 **Tibial characteristics**

186 High vs. low stocking density did not affect ($P > 0.05$) tibia length and width at 21 d, but both
187 traits were lowered ($P < 0.05$) at 35 d. Dietary CLA did not affect ($P > 0.05$) tibial length and
188 width at any age. The tibial breaking strength ranged from 14.3 kg to 15.0 kg at 21 d and from
189 30.9 kg to 31.9 kg at 35 d, but it was not affected by stocking density or dietary CLA. No
190 interaction between stocking density and CLA on the tibial traits was noted ($P > 0.05$) at any age
191 (Table 5).

192 **Ileal sIgA concentration**

193 To monitor the local immune response of broilers, ileal sIgA levels were assayed on 35 d (Table
194 6). However, none of the main factors (i.e., stocking density and CLA) affected ileal sIgA levels
195 in the broiler chickens, and no significant interactions were observed among the main factors
196 (Table 6)

197

198 **Serum biochemical indicators**

199 At 35 d, dietary CLA increased the GOT level by 26.1% compared to that in the control groups
200 fed a CLA-free diet (Table 7). The concentration of total cholesterol was elevated by 15.8% in
201 broilers fed on the CLA-enriched vs. -free diets, but the difference was not statistically significant
202 ($P = 0.074$). Dietary CLA did not affect concentrations of GPT, glucose, triglycerides, or uric acid
203 on 35 d (Table 7). In addition, none of the biochemical indicators were altered by stocking density.
204 No significant interaction was observed between the main factors and serum biochemical
205 parameters.

206

207 **Antioxidant indicators**

208 Antioxidant indicators, including superoxide dismutase, total antioxidant capacity, and
209 malondialdehyde, were not affected ($P > 0.05$) by stocking density or dietary CLA in broilers at
210 35 d (Table 8). No significant interactions between the main factors were noted for serum
211 antioxidant indicators.

212

213 **Immune and stress indicators**

214 The concentration of nitric oxide ranged from 12.7 to 15.2 μM at 21 d and from 20.5 to 24.7
215 μM at 35 d (Table 9), indicating the increased exposure of the host to external stimuli or potential
216 pathogens. However, neither stocking density nor dietary CLA affected the NO levels in the serum
217 samples of broiler chickens at any age. At 21 d, dietary CLA slightly decreased the concentration
218 of corticosterone in serum samples of broilers raised under low stocking density, but it was
219 increased in those raised under high stocking density, leading to a partial interaction between the
220 two factors ($P = 0.086$). Corticosterone concentrations ranged from 1,268 to 1,464 pg/mL at 35

221 d, but were not affected by stocking density or dietary CLA (Table 9).

222

223 **Fatty acid compositions of thigh meats**

224 Stocking density did not affect the fatty acid composition of the thigh meat of broilers at 35 d
225 (Table 10). Dietary CLA significantly increased the concentrations of myristic acid, palmitic acid,
226 stearic acid, and two CLA isomers (i.e., c9,t11 CLA and t10,c12 CLA) in thigh meat. In contrast,
227 broilers fed on CLA-enriched *vs.* -free diets had lower percentages of heptadecenoic acid, oleic
228 acid, linoleic acid, alpha-linolenic acid, dihomo-*r*-linolenic acid, arachidonic acid, nervonic acid,
229 and cervonic acid in their thigh meat of broiler chickens. No significant interactions were found
230 between stocking density and dietary CLA or fatty acid composition (Table 10).

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DISCUSSION

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232

233 Studies have revealed that the fatty acid composition of poultry meat can be altered by dietary
234 CLA [14,19] and stocking density [3], and that both dietary CLA [20,22] and stocking density [5]
235 can negatively affect broiler chicken production. We therefore conducted the current study to
236 investigate whether dietary CLA is equally incorporated into the meat of broiler chickens raised
237 under different stocking densities.

238 In this study, high vs. low stocking density improved the feed conversion ratio at the starter
239 phase but impaired feed intake and body weight gain at the finisher and whole phases in broiler
240 chickens. The adverse effects of high stocking density on growth performance in broiler chickens,
241 especially in the later days of production, have been well documented [24,5]. The reasons for the
242 high-density-mediated decrease in performance in the later days are attributed to the physical
243 difficulties that the chickens have in reaching feeders, and the lack of feeder space per bird [25].
244 In contrast, the improved feed conversion ratio in birds reared under high vs. low stocking
245 densities at the starter phase could be attributed to the provision of compatible space and low
246 maintenance energy with a high stocking density at this age [26]. Dietary CLA lowered the feed
247 intake but improved the feed conversion ratio in the starter phase compared to the control group
248 fed the CLA-free diet. This finding indicates that dietary CLA can help improve nutrient digestion
249 and absorption, especially in the early days, leading to the efficient utilization of absorbed
250 nutrients in broiler chickens. Dietary CLA increases body protein and fat deposition in mice [27],
251 pigs [28], and broiler chickens [19]. Our findings suggest that dietary CLA, as a functional feed
252 ingredient, can be added to the diet of broiler chickens without any apparent reduction in their
253 overall growth performance.

254 Body temperature can be affected by high stocking densities in chickens [25] and dietary CLA
255 in rodents [29]. However, the rectal temperature of the chickens was not affected by stocking
256 density or dietary CLA on 21 d or 35 d. An explanation for the lack of an effect of stocking density
257 and dietary CLA on body temperature is not readily available. The plausible explanation might

258 be related to stocking density employed in this study. In the present study, the high and low
259 stocking densities were set at 25.0 kg/m² and 15.0 kg/m² while those in Abudabos et al. [25]
260 ranged from 28.0 kg/m² to 40.0 kg/m². In any event, further studies are required to elucidate the
261 modulatory effects of dietary CLA in chickens exposed to heat or cold stress, as shown in
262 laboratory rodents [30].

263 A significant reduction in tibial length was observed in the high-vs. low-density groups;
264 however, this effect was only noted at 35 d. This finding is consistent with previous reports that
265 the tibia shortens with increasing stocking density, indicating that high density restricts the space
266 for movement, leading to weakened growth stimulation of the skeleton and increased skeletal
267 curvature [31,32]. Thus, the low tibial length observed in this study was likely secondary to the
268 stocking density-mediated decrease in body weight, especially during the later stages. Studies by
269 Henrique et al. [24] and Kelly et al. [33] indicated that CLA, especially the t10c12 isomer, has
270 the potential to enhance bone density by promoting bone formation and inhibiting bone resorption.
271 However, the tibial breaking strength was not altered by stocking density and dietary CLA at any
272 age, indicating that none of the factors might affect tibia mineralization.

273 Dietary CLA is known to enhance gut mucosal immunity by increasing sIgA levels in chickens
274 [18] and mice [34], whereas a high stocking density can negatively influence gut immunity by
275 decreasing sIgA levels in chickens [35]. No significant effects of stocking density or dietary CLA
276 on ileal sIgA levels were observed. A clear explanation of the discrepancy between our study and
277 that of Liu et al. [18] is not readily available as the inclusion levels of dietary CLA were equal in
278 both studies. However, Liu et al. [18] analyzed the jejunal mucosa at 21 d, whereas we used the
279 ileal mucosa at 35 d. Thus, further studies incorporating various gut segments from chickens of
280 different ages are needed to unravel the effects of dietary CLA on local immunity (e.g., sIgA).

281 Serum metabolic indicators serve as sensitive biomarkers for assessing internal homeostasis
282 and interpreting physiological states [36,37]. The findings of our study showed that dietary
283 supplementation with CLA significantly influenced hepatic metabolic conditions, which was
284 specifically manifested by a 26.1% increase in GOT activity. It is thus possible that elevated GOT
285 levels in serum samples can be interpreted as potential damage to the liver, heart, or muscles of

286 chickens [36,38] in response to dietary CLA. In line with our study, Attia et al. [39] reported that
287 dietary CLA at 20 and 40 g/kg significantly elevated GOT levels in serum samples. However, the
288 GOT levels observed in our study fell within the normal range (152–261 U/L) for chickens [40].
289 It is likely that the observed elevated GOT levels, although it remained within the physiological
290 range, might reflect subclinical metabolic stress induced by dietary CLA in hepatic or muscular
291 tissues. Thus, further histopathological evaluation is warranted to fully clarify the clinical
292 relevance of these biochemical fluctuations. Among the lipid parameters in the serum samples,
293 dietary CLA tended to increase total cholesterol by an average of 15.8% in broiler chickens, which
294 is consistent with the results of previous studies on broilers [41] and pigs [42]. In contrast, dietary
295 CLA was linked to lower cholesterol concentrations in the serum samples of broiler chickens by
296 inhibiting hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase [43]. Thus, studies have
297 determined inconsistent effects of dietary CLA on cholesterol metabolism in chickens, and this
298 therefore requires further study.

299 Overcrowded rearing conditions can induce stress responses in broiler chickens, leading to the
300 disruption of physiological functions [25]. Corticosterone serves as a key physiological indicator
301 of stress states [44] and contributes to the restoration of homeostatic balance through multiple
302 physiological regulatory mechanisms [45]. In the present study, a marginal interaction between
303 stocking density and dietary CLA, although not statistically significant, on serum corticosterone
304 concentrations was noted at 21 d ($P = 0.086$), but not at 35 d. Liu et al. [46] reported that dietary
305 CLA at 16 g/kg increased serum cortisol levels in laying hens. If our findings support those of Liu
306 et al. [18], then the CLA-mediated increase in the secretion of stress hormones (e.g.,
307 corticosterone or cortisol) is likely dependent on the stocking density in broiler chickens.
308 Nonetheless, the presence of a partial interaction between dietary CLA and stocking density at 21
309 d may require careful interpretation because of the absence of an effect of dietary CLA on
310 corticosterone at 35 d, and the lack of an effect of stocking density on corticosterone at 21 d and
311 35 d. The overall findings indicate that a high stocking density (25 birds/m²) did not induce a
312 significant increase in corticosterone, suggesting a potential physiological adaptation to crowding
313 or the involvement of other factors in HPA axis regulation. Similarly, dietary CLA exerted no

314 measurable effect on corticosterone, implying that its influence on stress physiology is unlikely
315 to be mediated through direct HPA axis modulation but rather through pathways related to lipid
316 metabolism and immune regulation.

317 We found that dietary CLA regulated fatty acid metabolism by increasing the percentage of
318 saturated fatty acids (i.e., myristic acid, palmitic acid, and stearic acid) and decreasing the
319 percentage of monounsaturated and polyunsaturated fatty acids (i.e., oleic acid, linolenic acid,
320 linolenic acid, and arachidonic acid) in the thigh meat of chickens. Our findings confirm those of
321 earlier studies showing the modulatory role of dietary CLA in fatty acid metabolism [47,20, 48].
322 In addition, a marked accumulation of CLA isomers within the muscle tissues was confirmed,
323 indicating that dietary CLA was equally incorporated into the thigh meat of broilers raised at high
324 and low stocking densities. The reduction in mono- and poly-unsaturated fatty acids noted in our
325 study can be attributed to the increase in fatty acid synthesis and/or the inhibition of hepatic Δ -5
326 and Δ -6 desaturases by dietary CLA [15,49,50]. This regulatory effect of dietary CLA on fatty
327 acid composition was independent of stocking density. Juniper et al. [3] reported that high vs. low
328 stocking density influenced the fatty acid composition of breast meat, but not thigh meat, in broiler
329 chickens. It is likely that dietary CLA, a potent inhibitor of fatty acid synthesis and desaturation,
330 plays a more dominant role in fatty acid metabolism than does stocking density, which led to the
331 absence of a stocking density effect in this study. In addition, stocking density, as an
332 environmental stressor, indirectly exerts its influence via neuroendocrine pathways (e.g.,
333 alterations in corticosterone levels), whereas dietary CLA directly influences the enzymatic
334 pathways of fatty acid synthesis and desaturation [15,51,32]. This difference in the action
335 mechanisms between stocking density and dietary CLA could explain the lack of significant
336 interaction effects between the two factors. Nevertheless, our study indicates that dietary CLA
337 can be effectively incorporated into thigh meat, regardless of stocking density.

CONCLUSION

338

339

340 In summary, dietary supplementation with CLA did not adversely affect growth performance,
341 rectal temperature, tibial mass, antioxidant status, or stress levels in both high and low stocking
342 density-raised broiler chickens. Dietary CLA altered the fatty acid profile of chicken muscle,
343 markedly increasing the proportion of saturated fatty acids while reducing unsaturated fatty acids
344 in the thigh meat of broiler chickens. Two isomers of CLA were only detected in CLA-fed broilers.
345 The CLA-mediated modulation of fatty acid metabolism and its incorporation into the chicken
346 meat were independent of stocking density, suggesting its applicability under both normal and
347 high-density rearing conditions without compromising physiological welfare indicators. It should
348 be, however, pointed out that the stocking density (25.0 kg/m² and 15.0 kg/m² for high and low
349 stocking density) used in this study is considered low compared to the industry standard of 39.0
350 kg/m², of which experimental setup might have influenced the effects of dietary CLA, if any, on
351 the parameters analyzed.

352

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355

356 **Declaration of interest**

357 No potential conflict of interest relevant to this article was reported.

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Tables and Figures

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527

528 **Table 1.** Ingredients and composition of experimental diets

Ingredients	Starter (g/kg)		Finisher (g/kg)	
	Control	CLA	Control	CLA
Corn, 8.8% CP	508	508	559.5	559.5
Soybean meal, 44.8% CP	277.4	277.4	239	239
Wheat, 11.5% CP	45	45	45	45
Corn gluten meal, 60% CP	87	87	60	60
Soybean oil	20	10	40	30
CLA	0	10	0	10
Salt	3	3	2	2
DCP	17.3	17.3	12.5	12.5
DL-methionine, 99%	1.7	1.7	1.2	1.2
L-lysine 78%	2.2	2.2	2.0	2.0
L-threonine	1.0	1.0	1.0	1.0
Cellulose	15	15	15	15
Limestone	13	13	14	14
NaHCO ₃	2.4	2.4	1.8	1.8
Choline chloride 50%	2.0	2.0	2.0	2.0
Vitamin Premix ¹	2.5	2.5	2.5	2.5
Mineral Premix ²	2.5	2.5	2.5	2.5
Total	1000	1000	1000	1000
Calculated nutrient composition				
AMEn (kcal/kg)	3,018	3,018	3,161	3,161
CP, %	23.0	23.0	20.1	20.1
Lysine, %	1.20	1.20	1.06	1.06
TSAA, %	0.93	0.93	0.78	0.86
Arginine, %	1.28	1.28	1.12	1.86
Calcium	1.02	1.02	0.93	0.91
NPP	0.45	0.45	0.35	0.35
ME:CP	131	131	158	158

529 ¹Vitamin premix provided following nutrients per kg of diet: vitamin A, 18000 IU; vitamin D3, 3000 IU;
 530 vitamin E, 80 IU; vitamin K3, 5 mg; vitamin B12, 0.06 mg; thiamin, 5 mg; riboflavin, 20 mg; pyridoxine,
 531 8 mg; niacin, 90 mg; biotin, 0.2 mg; folic acid, 1.1 mg; pantothenic acid, 50 mg.

532 ²Mineral premix provided the following nutrients per kg of diet: Fe, 80 mg; Mn, 40 mg; Zn, 12.5 mg; I,
 533 0.25 mg; Se, 0.2 mg; and Cu, 30 mg.

534 **Table 2.** Fatty acid composition of experimental diets

Ingredients ¹	Finisher (g/kg) ²	
	CON	CLA
C 12:0	0.27	0.28
C 14:0	0.20	0.18
C 16:0	12.7	10.9
C 18:0	3.48	3.21
C 18:1n9c	25.6	24.2
C 18:2n6c	52.1	43.2
C 20:0	0.32	NT ³
C 18:3n6	NT	0.27
C 18:3n3	3.61	2.66
c9,t11 CLA	NT	6.63
t10,c12 CLA	NT	6.61
Total	98.2	98.2

535 ¹C12:0 = lauric acid C14:0 = myristic acid; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1n9c = oleic
 536 acid; C18:2n6c = linoleic acid; C20:0 = arachidic acid; C18:3n3 = α -Linolenic acid; c9, t11 C18:2 = cis-9,
 537 trans-11 conjugated linoleic acid; c12, t10 C18:2 = cis-12, trans-10 conjugated linoleic acid.

538 ²CLA = add conjugated linoleic acid for 1% of diet; CON = control diet.

539 ³NT = not detected

540

541 **Table 3.** Effect of dietary conjugated linoleic acid on growth performance in broiler chickens raised under different stocking densities

		Feed intake (g/d/bird) ¹			Weight gain (g/d/bird)			Feed conversion ratio (g/g)		
		1-21d	21-35d	1-35d	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d
Density ²	Trt ²									
LSD	CON	47.7 ³	100.8	67.8	33.0	63.9	44.7	1.445	1.583	1.518
	CLA	44.7	106.8	68.4	32.2	66.2	45.1	1.392	1.613	1.516
HSD	CON	45.1	90.0	62.6	33.3	53.7	41.3	1.353	1.681	1.519
	CLA	43.7	91.6	62.4	32.7	55.6	41.6	1.339	1.650	1.500
SEM ⁴		0.97	2.33	1.03	0.71	1.46	0.80	0.015	0.041	0.023
Main factors										
LSD		46.2	103.8 ^a	68.1 ^a	32.6	65.0 ^a	44.9 ^a	1.424 ^a	1.598	1.517
HSD		44.4	90.8 ^b	62.5 ^b	33.0	54.7 ^b	41.4 ^b	1.356 ^b	1.666	1.509
	CON	46.4 ^A	95.4	65.2	33.2	58.8	43.0	1.407 ^A	1.632	1.518
	CLA	44.2 ^B	99.2	65.4	32.4	60.9	43.4	1.376 ^B	1.631	1.508
P-value										
Density (D)		0.078	<.0001	<.0001	0.557	<.0001	<.0001	<.0001	0.108	0.747
Trt (T)		0.038	0.118	0.850	0.313	0.157	0.622	0.035	0.986	0.658
D × T		0.417	0.348	0.724	0.894	0.869	0.955	0.207	0.458	0.733

542 ¹ADFI = average daily feed intake; ADWG = average daily weight gain; FCR = feed conversion ratio.

543 ²CLA = added conjugated linoleic acid at 1% of diet; CON = control diet; LSD = low stocking density; HSD = high stocking density.

544 ³Values are means of pen-level data (n = 7 pens per treatment).

545 ⁴SEM = pooled standard error of the means.

546 ⁵Values within a column bearing different uppercase superscript letters (A, B for stocking density; A, B for dietary treatment) are significantly different ($P < 0.05$).

547 **Table 4.** Effect of dietary conjugated linoleic acid on rectal temperature in broiler
 548 chickens raised under different stocking densities

		Rectal temperature (°C)	
		21 d	35 d
Density ¹	Trt ¹		
LSD	CON	41.03 ²	40.90
	CLA	41.10	41.31
HSD	CON	40.90	41.31
	CLA	41.09	41.34
SEM ³		0.13	0.16
Main factors			
LSD		41.06	41.10
HSD		40.99	41.33
	CON	40.96	41.10
	CLA	41.09	41.33
<i>P</i> -value			
Density (D)		0.587	0.185
Trt (T)		0.331	0.185
D × T		0.663	0.263

549 ¹CLA = conjugated linoleic acid added to 1% of diet; CON = control diet; LSD = low stocking density;
 550 HSD = high stocking density.

551 ²Values are means of pen-level data (n = 7 pens per treatment).

552 ³SEM = pooled standard error of the means.

553 ⁴No significant main effects or interactions were observed ($P < 0.05$).

554 **Table 5.** Effect of dietary conjugated linoleic acid on tibial characteristics in broiler
 555 chickens raised under different stocking densities

		Tibial length		Tibial thickness		Tibial breaking str	
		(mm)		(mm)		ength (kg)	
		21d	35d	21d	35d	21d	35d
Density ¹	Trt ¹						
LSD	CON	74.71 ²	101	5.86	8.29	14.89	30.97
	CLA	71.86	101.43	5.29	8.29	13.78	32.90
HSD	CON	73.71	99.71	5.57	8.00	15.15	31.51
	CLA	73.43	98.14	5.43	7.71	14.90	30.26
SEM ³		1.01	0.75	0.21	0.22	1.33	2.53
Main factors							
LSD		73.29	101.21 ^a	5.57	8.29	14.33	31.93
HSD		73.57	98.93 ^b	5.50	7.86	15.02	30.89
	CON	74.21	100.36	5.71	8.14	15.02	31.24
	CLA	72.64	99.79	5.36	8.00	14.34	31.58
<i>P</i> -value							
Density (D)		0.780	0.005	0.742	0.066	0.608	0.684
Trt (T)		0.133	0.452	0.109	0.526	0.613	0.894
D × T		0.215	0.194	0.327	0.526	0.751	0.535

556 ¹CLA = conjugated linoleic acid added to 1% of diet; CON = control diet; LSD = low stocking density;
 557 HSD = high stocking density.

558 ²Values are means of pen-level data (n = 7 pens per treatment).

559 ³SEM = pooled standard error of the means.

560 ⁴Values within a column bearing different uppercase superscript letters(a and b) are significantly different
 561 (*P* < 0.05).

562 **Table 6.** Effect of dietary conjugated linoleic acid on secretory immunoglobulin A (sIgA)
 563 contents in ileal mucosa of broiler chickens raised under different stocking densities

		sIgA ($\mu\text{g}/\text{mg}$ of protein)
Density ¹	Trt ¹	
LSD	CON	0.271 ²
	CLA	0.283
HSD	CON	0.292
	CLA	0.259
SEM ³		0.032
Main factors		
LSD		0.277
HSD		0.277
	CON	0.282
	CLA	0.271
<i>P</i> -value		
Density (D)		0.998
Trt (T)		0.732
D \times T		0.507

564 ¹CLA = conjugated linoleic acid added to 1% of diet; CON = control diet; LSD = low stocking density;
 565 HSD = high stocking density.

566 ²Values are means of pen-level data (n = 7 pens per treatment).

567 ³SEM = pooled standard error of the means.

568 ⁴No significant main effects or interactions were observed ($P < 0.05$).

569 **Table 7.** Effect of dietary conjugated linoleic acid on serum biochemical parameters in
 570 broiler chickens raised under different stocking densities

		GOT ¹ (U/L)	GPT (U/L)	GLU (mg/dL)	TCHO (mg/dL)	TG (mg/dL)	UA (mg/dL)
Density ²	Trt ²						
LSD	CON	162.6 ³	4.57	210.9	71.9	82.6	5.81
	CLA	189.0	4.57	176.0	75.1	77.0	7.63
HSD	CON	155.4	4.43	203.7	63.6	54.3	4.90
	CLA	212.0	4.86	213.7	81.7	80.1	5.79
SEM ⁴		14.6	0.29	33.5	5.7	21.8	1.12
Main factors							
LSD		175.8	4.57	193.4	73.5	79.8	6.72
HSD		183.7	4.64	208.7	72.6	67.2	5.34
	CON	159.0 ^B	4.50	207.3	67.7	68.4	5.36
	CLA	200.5 ^A	4.71	194.9	78.4	78.6	6.71
<i>P</i> -value							
Density(D)		0.592	0.807	0.652	0.882	0.570	0.231
Trt(T)		0.009	0.465	0.714	0.074	0.647	0.240
D*T		0.313	0.465	0.509	0.207	0.479	0.682

571 ¹GLU = glucose; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; TCHO
 572 = total cholesterol; TG = triglyceride; UA = uric acid

573 ²CLA = added conjugated linoleic acid at 1% of diet; CON = control diet; LSD = low stocking density;
 574 HSD = high stocking density.

575 ³Values are means of pen-level data (n = 7 pens per treatment).

576 ⁴SEM = pooled standard error of the means.

577 ⁵Values within a column bearing different uppercase superscript letters(A and B) are significantly different
 578 (*P* < 0.05).

579

580 **Table 8.** Effect of dietary conjugated linoleic acid on antioxidant indicators in serum
 581 samples of broiler chickens raised under different stocking densities

		SOD ¹ , (%)	TAC, mM	MDA, nM
Density ²	Trt ²			
LSD	CON	81.93 ³	210.2	3.14
	CLA	90.51	178.6	2.99
HSD	CON	87.98	209.4	3.16
	CLA	89.77	192.4	2.97
SEM ⁴		5.02	52.2	0.11
Main factors				
LSD		86.22	194.4	3.07
HSD		88.88	200.9	3.07
	CON	84.96	209.8	3.15
	CLA	90.14	185.5	2.98
<i>P</i> -value				
Density (D)		0.601	0.902	0.993
Trt (T)		0.312	0.646	0.135
D × T		0.506	0.890	0.866

582 ¹SOD = superoxide dismutase; MDA = malondialdehyde; TAC = total antioxidant capacity.

583 ²CLA = added conjugated linoleic acid at 1% of diet; CON = control diet; LSD = low stocking density;
 584 HSD = high stocking density.

585 ³Values are means of pen-level data (n = 7 pens per treatment).

586 ⁴SEM = pooled standard error of the means.

587 ⁵No significant main effects or interactions were observed (*P* < 0.05).

588 **Table 9.** Effect of dietary conjugated linoleic acid on concentrations of nitric oxide and
 589 corticosterone in serum samples of broiler chickens raised under different stocking
 590 densities

		Nitric oxide (μM)		Corticosterone (pg/mL)	
		21d	35d	21d	35d
Density ¹	Trt ¹				
LSD	CON	14.17 ²	23.9	406	1464
	CLA	15.17	21.3	332	1316
HSD	CON	14.49	20.5	274	1268
	CLA	12.74	24.7	546	1424
SEM ³		1.63	2.34	90	276
Main factors					
LSD		14.67	22.6	365	1397
HSD		13.62	22.6	410	1359
	CON	14.33	22.2	329	1375
	CLA	13.96	23.0	447	1379
<i>P</i> -value					
Density (D)		0.525	0.998	0.650	0.904
Trt (T)		0.820	0.731	0.221	0.976
D \times T		0.407	0.155	0.086	0.626

591 ¹CLA = conjugated linoleic acid added to 1% of diet; CON = control diet; LSD = low stocking density;
 592 HSD = high stocking density.

593 ²Values are means of pen-level data (n = 7 pens per treatment).

594 ³SEM = pooled standard error of the means.

595 ⁴No significant main effects or interactions were observed ($P < 0.05$).

596 **Table 10.** Effect of dietary conjugated linoleic acid (CLA) on fatty acid composition (% of total fatty acid methyl esters) of thigh meats in broiler
 597 chickens raised under different stocking densities

		C14:0 ¹	C16:0	C16:1	C17:1	C18:0	C18:1n9c	C18:2n6c	C18:3n3	CLA c9,t11	CLA t10,c12	C22:0	C20:3n6	C20:4n6	C24:1n9	C22:6n3
Density ²	Trt ²															
LSD	CON	0.46 ³	20.04	2.09	0.54	8.94	24.88	28.62	1.65	NT ⁴	NT	0.61	0.70	3.31	0.87	0.29
	CLA	0.68	25.28	1.66	0.25	12.31	18.83	26.84	1.37	1.86	1.09	0.48	0.45	1.87	0.46	0.17
HSD	CON	0.51	19.77	2.21	0.54	8.73	25.06	29.37	1.74	NS	NS	0.60	0.61	3.42	0.90	0.31
	CLA	0.62	24.66	1.71	0.31	11.86	19.74	27.18	1.36	1.66	0.96	0.51	0.45	2.10	0.54	0.20
SEM ⁴		0.07	0.75	0.77	0.08	0.79	1.38	1.19	0.11	0.11	0.07	0.15	0.11	0.40	0.12	0.06
Main factors																
LSD		0.57	22.66	1.96	0.43	10.63	21.86	27.73	1.50	0.93	0.55	0.54	0.56	2.59	0.66	0.24
HSD		0.57	22.22	1.88	0.40	10.29	22.40	28.27	1.53	0.83	0.48	0.55	0.55	2.76	0.72	0.26
	CON	0.48 ^B	19.91 ^B	2.15	0.54 ^A	8.83 ^B	24.97 ^A	28.99 ^A	1.69 ^A	NT ⁵	NT	0.61	0.65 ^A	3.37 ^A	0.88 ^A	0.30 ^A
	CLA	0.65 ^A	24.97 ^A	1.68	0.28 ^B	12.09 ^A	19.29 ^B	27.01 ^B	1.36 ^B	1.76	1.02	0.50	0.45 ^B	1.99 ^B	0.50 ^B	0.19 ^B
P-value																
Density(D)		0.96	0.41	0.82	0.59	0.53	0.55	0.37	0.55	0.37	0.32	0.84	0.43	0.65	0.49	0.43
Trt (T)		<.0001	<.0001	0.20	<.0001	<.0001	<.0001	<.005	<.0001	<.0001	<.0001	0.15	<.005	<.001	<.0001	<.005
D*T		0.21	0.74	0.91	0.55	0.82	0.68	0.73	0.42	0.37	0.32	0.79	0.46	0.87	0.80	0.80

598 ¹C14:0 = myristic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C17:1 = cis-10-heptadecenoic acid; C18:0 = stearic acid; C18:1n9c = oleic acid; C18:2n6c = linoleic
 599 acid; C18:3n3 = α -Linolenic acid; c9, t11 C18:2 = cis-9, trans-11 conjugated linoleic acid; c12, t10 C18:2 = cis-12, trans-10 conjugated linoleic acid; C22:0 = behenic acid;
 600 C20:3n6 = cis-8,11,14-Eicosatrienoic acid; C20:4n6 = arachidonic acid; C24:1n9 = nervonic acid; C22:6n3 = Docosahexaenoic acid.

601 ²CLA = added conjugated linoleic acid at 1% of diet; CON = control diet; LSD = low stocking density; HSD = high stocking density.

602 ³Values are means of pen-level data (n = 7 pens per treatment).

603 ⁴SEM = pooled standard error of the means.

604 ⁵NT = not detected.

605 ⁶Values within a column bearing different uppercase superscript letters (A, B for stocking density; A, B for dietary treatment) are significantly different ($P < 0.05$).

ACCEPTED