# Effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens

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**ABSTRACT** The objective of the current experiment was to investigate the effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens. The experiment was conducted in a completely randomized design with a 2 × 4 factorial arrangement consisting of sex and four different stocking densities in battery cages. A total of 540 1-d-old Ross 308 broiler chickens were allotted to one of eight treatments with five replicates. Within each sex, birds were raised at four different stocking densities of 15.2, 20.2, 25.3, or 30.4 birds/$m^2$ from 1 to 28 d of age. Different stocking densities were achieved by raising a different number of birds per battery cage with identical floor size (0.76 m × 0.78 m). At the end of the experiment, two birds per replicate were euthanized by $CO_2$ asphyxiation to collect tissue samples for further analyses. Results indicated that no interactions between sex and stocking density were observed for all measurements except for serum lipopolysaccharide (LPS) concentrations. Increasing stocking density decreased (linear, $P < 0.01$) body weight gain and feed intake, but had no negative effects on meat quality. Transepithelial electrical resistance values, a measure of intestinal permeability, were decreased (linear, $P < 0.01$) with increasing stocking density, regardless of sex. Accordingly, serum LPS concentrations were increased (linear, $P < 0.01$) with increasing stocking density. However, increasing stocking density increased serum LPS concentrations in male broiler chickens, but had no effects on female broiler chickens, showing an interaction ($P < 0.01$). The expression of zonula occludens-1 (*ZO-1*) and junctional adhesion molecule B (*JAM-2*) was decreased (linear, $P < 0.05$) with increasing stocking density. In conclusion, increasing stocking density decreases broiler performance regardless of sex and this negative effect is likely associated with decreased intestinal barrier function.

**Key words:** broiler chicken, intestinal barrier function, sex, stocking density, tight junction-related gene expression

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## INTRODUCTION

Stocking density is an important issue in the poultry industry because it is highly related to the outcome of poultry productivity as well as animal welfare issues (Zhang et al., 2013). Stocking density for broiler chickens is defined as the number of birds or the total live weight of birds in a fixed space (Estevez, 2007). Therefore, high stocking density increases profitability due to a higher production of chicken meat per space (Puron et al., 1995). However, if stocking density exceeds over the proper range, the productivity is rather decreased because of increased health problems and decreased performance of broiler chickens (Estevez, 2007). Likewise, it has been documented that high stocking density decreased body weight (**BW**), feed intake (**FI**), and feed efficiency (**FE**) in broiler chickens (Zuowei et al., 2011; Sun et al., 2013; Cengiz et al., 2015). Moreover, high stocking density was reported to decrease the absorptive capacity by impairing villus structures of the small intestine in broiler chickens (Shakeri et al., 2014; Li et al., 2017). However, physiological alterations in the gastrointestinal tract (**GIT**) of broiler chickens due to high stocking density in relation to productive performance have not been examined.

Intestinal barrier plays a key role in maintaining the health of animals because the GIT is always exposed to harmful environmental agents (Elphick and Mahida, 2005). For intestinal barrier, intestinal mucosa layers work as the first line of defense against the invasion of pathogenic microorganisms and antigens in the lumen of the GIT (Elphick and Mahida, 2005). The defensive mechanisms of intestinal mucosa layers include mucous as a chemical defense and tight junction as a mechanical defense (Song et al., 2017). Recently, the barrier function of tight junction in the GIT has gained increasing attention for its role in the health of animals because if tight junction barrier is impaired,


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inflammatory responses are increased in the GIT, which subsequently decreases animal health and productivity (Lambert, 2009). It has been reported that decreased health and production of animals exposed to heat stress is highly associated with impaired barrier function of tight junction (Lambert, 2009; Song et al., 2014; Shin et al., 2018). However, few data are available regarding the effects of stocking density on intestinal barrier function in broiler chickens.

Male broiler chickens are assumed to require more space than female broiler chickens at the same age because male chickens grow faster than female chickens (Puron et al., 1995). However, Estevez (2007) reported that the negative effects of stocking density were similar between sexes. On the contrary, Zuowei et al. (2011) reported that female chickens required more space than male chickens at a similar BW near to marketing age. Although the relationship between sex and stocking density is controversial, the effects of stocking density for broiler chickens on intestinal barrier function may depend on the sex. However, this hypothesis has not been tested previously.

The objective of the current experiment, therefore, was to investigate the effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens.

## MATERIALS AND METHODS

### Birds, Experimental Design, and Management

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee at Chung-Ang University. The experiment was conducted in a completely randomized design with a 2 × 4 factorial arrangement with sex and four different stocking densities in battery cages. A total of 540 1-d-old male and female Ross 308 broiler chickens were allotted to one of eight treatments with five replicates. Within each sex, four different stocking densities were achieved by raising a different number of birds per battery cage with identical floor size (0.76 m × 0.78 m). Four different stocking densities included 15.2 birds/$m^2$ (9 birds/cage), 20.2 birds/$m^2$ (12 birds/cage), 25.3 birds/$m^2$ (15 birds/cage), or 30.4 birds/$m^2$ (18 birds/cage). Birds were raised from 1 to 28 d of age. In addition, extra cages were used to maintain the designed stocking density. If decreased birds were found, extra birds with the similar BW to the deceased birds were used as a replacement to maintain the stocking density until 21 d of the experimental period. Replaced birds were identified with a leg band, and therefore, those birds were not used for sample collections. Two galvanized iron troughs (0.76 m long × 0.12 m wide × 0.08 m depth) hanging each outside of the cage were used for a feeder and a drinker. Therefore, the space of the feeder and drinker was not included to calculate the stocking density. Two-tiered battery cages with wire floors were used. All battery cages were placed in an environmentally controlled room. Each replicate was randomly allotted to 1 of 40 battery cages. Room temperature was maintained at 32°C during the first wk and reduced gradually until a constant temperature of 24°C was achieved. A 24-h lighting schedule was used during the entire experiment. The same commercial diet ($AME_n$, 3130 kcal/kg; CP, 21.0%; Ca, 0.90%; available P, 0.38%; Lys, 1.33%; Met + Cys, 0.98%) was fed to all birds throughout the experiment. The diet and water were provided ad libitum for 28 d. The body weight gain (**BWG**) and FI were recorded at the end of the experiment. The uniformity of BW was calculated based on the CV of the final BW. The FE was calculated as BWG divided by FI after correcting for mortality (Kim et al., 2017).

### Sample Collection and Chemical Analysis

At the end of the experiment, 2 birds per replicate with BW close to the average of each cage were euthanized by $CO_2$ asphyxiation. One bird was used for analyzing meat quality, serum lipopolysaccharide (**LPS**), and jejunal tight junction-related gene expression, whereas the other bird was used for analyzing intestinal permeability using the Ussing chamber.

Blood samples were collected by heart puncture using a 6.0 ml BD Vacutainer serum tube (BD, Franklin Lakes, NJ). Blood samples were then centrifuged at 3,000 × g at 4°C for 20 min to separate the serum and were stored at –20°C before LPS analysis. The serum LPS concentrations were analyzed using a Microplate Reader (Molecular Device, Sunnyvale, CA) and a commercial chicken LPS ELISA Kit (MyBiosource, San Diego, CA). The enzyme-linked immunosorbent antibody assay procedure was carried out according to the protocol of the manufacturer, and absorbance was measured at 450 nm. Mucosal samples from the jejunum of the small intestine were collected by the gentle scraping, frozen in liquid nitrogen, and stored at –85°C until gene expression was analyzed. For meat quality, the right portion of breast meat was used for analyzing pH at 1 and 24 h postmortem using a pH meter (Hanna Instruments, Nusfalau, Romania) and meat color at 24 h postmortem according to the CIE color scale (Minolta Chroma Meter CR-400, Osaka, Japan) for lightness ($L^*$), redness ($a^*$), and yellowness ($b^*$). The left portion of the breast meat was used to measure water holding capacity (**WHC**) at 24 h postmortem and the thiobarbituric acid reactive substance (**TBARS**) value at 7 d after storage at 4°C as described by Kim et al. (2012).

The analysis of tight junction-related gene expression in the jejunal mucosa followed the method of Shin et al. (2018). In short, total RNA was extracted from the jejunal mucosa using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Gene expression was examined for zonula occludens-1

**Table 1.** Primers used for quantitative RT-PCR.

| Primer name[1] | Primer sequence[2] 5′-3′ | Tm[3], °C | Product size, bp | GenBank accession number |
|---|---|---|---|---|
| *GAPDH* | F: TGCTGCCCAGAACATCATCC<br>R: ACGGCAGGTCAGGTCAACAA | 50.0 - 65.0 | 142 | NM_204305 |
| *ZO-1* | F: AATACCTGACTGTCTTGCAG<br>R: TAAAGAAGGCTTTCCCTGAC | 58.3 | 145 | XM_015278975.1 |
| *OCLN* | F: TCGTGCTGTGCATCGCCATC<br>R: CGCTGGTTCACCCCTCCGTA | 60.0 | 178 | NM_205128.1 |
| *CLDN-1* | F: CAGACTCTAGGTTTTGCCTT<br>R: AATCTTTCCAGTGGCGATAC | 58.3 | 149 | NM_001013611.2 |
| *JAM-2* | F: GTGAATTTACAGTTCCTCCC<br>R: TCCTGTCTTTTCCAGTAAGG | 53.9 | 158 | NM_0,010,06257.1 |

[1] *GAPDH*, glyceraldehyde-3-phosphate; *ZO-1*, zonula occludens-1; *OCLN*, occludin; *CLDN-1*, claudin-1; *JAM-2*, junctional adhesion molecules-B.
[2] F, forward; R, reverse.
[3] Tm, melting temperature.

(***ZO-1***), occludin (***OCLN***), claudin-1 (***CLDN-1***), and junctional adhesion molecule B (***JAM-2***). A quantitative RT-PCR was performed based on the general RT-PCR method (LightCycler 96 system, Roche, Basel, Switzerland). Gene-specific primers for target genes were designed using NCBI/Primer-BLAST and the specificity of the primers was confirmed by PCR amplification as demonstrated by Aznar and Alarcón (2002). The primer sequences and amplification temperatures are listed in Table 1. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Thomsen et al., 2010).

Intestinal permeability was determined by transepithelial electrical resistance (**TER**) value in a dual-channel self-contained Ussing chamber system (U2500, Warner Instruments, Hamden, CT). The mid-jejunum tissue samples (e.g., between the end of duodenal loop and Meckel's diverticulum) were immediately excised from the birds and placed into chilled and aerated Krebs-Henseleit buffer at pH 7.4. The jejunum was cut into sections of 3 to 5 cm, and adhering fat and mesenteries were removed. The jejunal samples were subsequently placed vertically between the two halves of Ussing chamber as described in detail by Ruhnke et al. (2013).When the samples were clamped in the Ussing chamber, the samples were continuously aerated with 95% $O_2$ and 5% $CO_2$ mixture, and completely immersed in Krebs buffer. The buffer was continuously stirred and heated to 38°C to 39°C. After the stabilization process (about 10 min) of the Ussing chamber and samples, short-circuit currents and epithelial voltages were recorded about every 10 min. The TER values were expressed as $\Omega/cm^2$. After 20 min of operation, TER values were calculated by averaging the current during the second 10 min of tissue stabilization according to Ohm's law (Gabler et al., 2007).

### Statistical Analysis

All data were analyzed by 2-way analysis of variance in a completely randomized design with the MIXED procedure of SAS (SAS Institute Inc. Cary, NC). The replicate was considered as an experimental unit for all analyses. The model included the effects of sex, stocking density, and their interaction. Outliers were checked using the UNIVARIATE procedure of SAS. Preplanned orthogonal polynomial contrast test was performed to examine linear and quadratic effects of increasing stocking density on all measurements, regardless of sex (Seo et al., 2018). The one-slope broken-line also was conducted to predict the upper limits of stocking density in broiler chickens (Robbins et al., 2006); however, no breaking points were generated for all measurements. Moreover, an additional model with only stocking density as a class variable was used to identify critical stocking density by comparing means using PDIFF option of SAS (Buijs et al., 2009). Significance for statistical test was set at $P < 0.05$.

## RESULTS

### Growth Performance

No interaction between sex and stocking density for growth performance was observed (Table 2). During the overall experimental period, however, male broiler chickens had greater ($P < 0.05$) FE and CV of BW (i.e., low uniformity of BW) compared with female broiler chickens. Regardless of sex, increasing stocking density decreased (linear, $P < 0.01$) BWG and FI of broiler chickens. In particular, birds raised at the stocking density of 30.4 birds/$m^2$ had less ($P < 0.05$) BWG than those raised at other stocking densities.

### Breast Meat Quality

There was no interaction for breast meat quality between sex and stocking density (Table 3). However, female broiler chickens had greater ($P < 0.05$) breast meat yield than male broiler chickens. However, other measurements including pH, WHC, meat color, and TBARS were not affected by sex and stocking density.

**Table 2.** Effects of stocking density and sex on growth performance of broiler chickens.

| Item | | Growth performance[1] | | | |
|---|---|---|---|---|---|
| | | BWG, g | FI, g | FE, g/g | CV of final BW, % |
| Sex | SD[2] | | | | |
| Male | 15.2 | 1,418 | 2,057 | 689 | 14.4 |
| | 20.2 | 1,294 | 1,878 | 689 | 17.2 |
| | 25.3 | 1,402 | 2,011 | 697 | 16.4 |
| | 30.4 | 1,271 | 1,838 | 693 | 16.5 |
| Female | 15.2 | 1,348 | 1,969 | 685 | 14.0 |
| | 20.2 | 1,310 | 1,944 | 674 | 12.0 |
| | 25.3 | 1,345 | 1,961 | 685 | 12.0 |
| | 30.4 | 1,168 | 1,773 | 659 | 13.9 |
| SEM (n = 5) | | 39.5 | 53.3 | 11.0 | 1.70 |
| Main effect | | | | | |
| Sex | | | | | |
| Male | | 1,346 | 1,946 | 691 | 16.1 |
| Female | | 1,293 | 1,912 | 676 | 13.0 |
| SEM (n = 20) | | 19.8 | 26.6 | 5.5 | 0.85 |
| SD | | | | | |
| 15.2 | | 1,383[a] | 2,013[a] | 687 | 14.2 |
| 20.2 | | 1,302[b] | 1,911[a,b] | 681 | 14.6 |
| 25.3 | | 1,373[a,b] | 1,986[a] | 691 | 14.2 |
| 30.4 | | 1,219[c] | 1,805[b] | 676 | 15.2 |
| SEM (n = 10) | | 27.9 | 37.7 | 7.8 | 1.20 |
| Effect (*P*-value) | df | | | | |
| Sex | 1 | 0.066 | 0.372 | 0.049 | 0.013 |
| SD | 3 | <0.001 | 0.002 | 0.531 | 0.923 |
| Sex × SD | 3 | 0.501 | 0.482 | 0.574 | 0.526 |
| SD (linear)[3] | 1 | 0.002 | 0.003 | 0.490 | 0.637 |
| SD (quadratic)[3] | 1 | 0.202 | 0.306 | 0.537 | 0.802 |

[a–c]Means in the same column with different superscripts are different ($P < 0.05$).

[1]BWG, body weight gain; FI, feed intake; FE, feed efficiency (BWG: FI); CV, coefficients of variation.

[2]SD, stocking density (birds/m$^2$).

[3]Linear and quadratic effects of increasing stocking density with the pooled data from male and female chickens.

### *Intestinal Permeability and Serum LPS Concentration*

No interaction between sex and stocking density for intestinal permeability measured by the Ussing chamber was observed (Table 4). The TER values were not different between sexes but increasing stocking density decreased (linear, $P < 0.01$) TER values, which indicates that intestinal permeability was increased by increasing stocking density. Likewise, increasing stocking density increased (linear, $P < 0.01$) serum LPS concentrations, which is known to be increased when intestinal permeability is increased. However, there was an interaction ($P < 0.01$) for serum LPS concentrations between sex and stocking density because increasing stocking density had no effects on serum LPS concentrations in female broiler chickens, but increased serum LPS concentrations in male broiler chickens. The TER value for birds raised at the stocking density of 30.4 birds/m$^2$ was less ($P < 0.05$) than those raised at the stocking density of 15.2 or 20.2 birds/m$^2$. Likewise, birds raised at the stocking density of 30.4 birds/m$^2$ had greater ($P < 0.05$) serum LPS concentrations than those raised at other stocking densities.

### *Tight Junction-related Gene Expression*

The interaction between sex and stocking density was not significant for selected tight junction-related gene expression (Table 5). However, increasing stocking density decreased the expression of *ZO-1* (linear, $P < 0.05$) and *JAM-2* (linear, $P < 0.01$). The expression of *JAM-2* for birds raised at the stocking density of 30.4 birds/m$^2$ was less ($P < 0.05$) than those raised at the stocking density of 15.2 birds/m$^2$. However, the expression of *OCLN* and *CLDN-1* was not influenced by sex and stocking density.

## DISCUSSION

Stocking density for broiler chickens is defined as the number of birds or the total live weight of birds in a fixed space (Estevez, 2007). Most of previous experiments created different stocking densities by raising a different number of birds in pens or cages of an identical floor space. However, this experimental procedure can have a limitation because the effects of stocking density are confused with the effects of group size (Buijs et al., 2009). As an alternative way, it is possible to produce different stocking densities by raising the same number of birds in different floor spaces, which can exclude the effects of group size. However, this alternative procedure appeared to be less applicable to the commercial situation. Therefore, from a practical standpoint, different stocking densities were generated in the current experiment by raising a different number of birds in battery cages with an identical floor space.

Increasing stocking density decreased BWG and FI in the current experiment. This result agrees with previous experiments that reported high stocking density decreased growth performance of broiler chickens compared with low stocking density (Dozier et al., 2006; Shakeri et al., 2014; Cengiz et al., 2015). The reason for these observations has been associated with various environmental and behavioral factors. High stocking density disturbs the birds' movements in a given space, and therefore, the birds raised at a high stocking density have more difficulty accessing to feeders and drinkers (Cengiz et al., 2015). In addition, Feddes et al. (2002) reported that birds raised at a high stocking density experienced with moderate heat stress because of a reduction in heat exchange as a result of crowding. Moreover, high stocking density also decreased the floor litter quality, leading to a foodpad and hock problem, although the problem is not often observed in battery cages. Our results indicate that the stocking density of up to 25.3 birds/m$^2$ may have no detrimental effects on growth performance of broiler chickens. It is likely, however, that the stocking density of 30.4 birds/m$^2$ decreases growth performance of broiler chickens raised in battery cages. This result disagrees with previous experiments reporting that high stocking density of over 16 or 19 birds/m$^2$ in

**Table 3.** Effects of stocking density and sex on breast meat quality of broiler chickens.

| Item | | Breast meat quality[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Yield, % | pH, 1h | pH, 24h | WHC, % | L* | a* | b* | TBARS |
| Sex | SD[2] | | | | | | | | |
| Male | 15.2 | 17.5 | 6.43 | 5.79 | 78.8 | 49.4 | 6.2 | 9.3 | 0.234 |
| | 20.2 | 17.6 | 6.63 | 5.80 | 79.2 | 50.8 | 5.0 | 10.0 | 0.220 |
| | 25.3 | 17.8 | 6.67 | 5.93 | 83.8 | 49.0 | 4.1 | 8.3 | 0.211 |
| | 30.4 | 16.8 | 6.52 | 5.85 | 78.0 | 50.7 | 5.3 | 9.9 | 0.197 |
| Female | 15.2 | 19.1 | 6.66 | 5.91 | 83.3 | 48.9 | 5.3 | 9.4 | 0.182 |
| | 20.2 | 18.5 | 6.54 | 5.87 | 80.7 | 49.7 | 4.7 | 9.0 | 0.195 |
| | 25.3 | 18.4 | 6.59 | 5.84 | 78.5 | 49.4 | 4.8 | 9.8 | 0.210 |
| | 30.4 | 17.6 | 6.73 | 5.82 | 80.5 | 51.5 | 5.0 | 10.2 | 0.196 |
| SEM (n = 5) | | 0.54 | 0.077 | 0.063 | 1.96 | 0.99 | 0.57 | 0.54 | 0.018 |
| Main effect | | | | | | | | | |
| Sex | | | | | | | | | |
| Male | | 17.5 | 6.56 | 5.84 | 80.0 | 50.0 | 5.2 | 9.4 | 0.215 |
| Female | | 18.4 | 6.63 | 5.86 | 80.7 | 49.9 | 4.9 | 9.6 | 0.196 |
| SEM (n = 20) | | 0.27 | 0.038 | 0.031 | 0.98 | 0.50 | 0.28 | 0.27 | 0.009 |
| SD | | | | | | | | | |
| 15.2 | | 18.3 | 6.54 | 5.85 | 81.1 | 49.1 | 5.7 | 9.3 | 0.208 |
| 20.2 | | 18.1 | 6.59 | 5.84 | 79.9 | 50.3 | 4.8 | 9.5 | 0.208 |
| 25.3 | | 18.1 | 6.63 | 5.89 | 81.1 | 49.2 | 4.5 | 9.1 | 0.210 |
| 30.4 | | 17.2 | 6.62 | 5.83 | 79.3 | 51.1 | 5.2 | 10.0 | 0.196 |
| SEM (n = 10) | | 0.38 | 0.054 | 0.044 | 1.38 | 0.70 | 0.40 | 0.38 | 0.013 |
| Effect (*P*-value) | df | | | | | | | | |
| Sex | 1 | 0.018 | 0.211 | 0.656 | 0.574 | 0.882 | 0.606 | 0.636 | 0.146 |
| SD | 3 | 0.213 | 0.642 | 0.808 | 0.736 | 0.172 | 0.191 | 0.354 | 0.862 |
| Sex × SD | 3 | 0.808 | 0.066 | 0.324 | 0.090 | 0.749 | 0.555 | 0.161 | 0.484 |
| SD (linear)[3] | 1 | 0.073 | 0.243 | 0.952 | 0.510 | 0.130 | 0.270 | 0.331 | 0.578 |
| SD (quadratic)[3] | 1 | 0.359 | 0.634 | 0.656 | 0.792 | 0.611 | 0.063 | 0.288 | 0.606 |

[1]WHC, water holding capacity; L*, lightness; a*, redness; b*, yellowness; TBARS, thiobarbituric acid reactive substances.

[2]SD, stocking density (birds/m$^2$).

[3]Linear and quadratic effects of increasing stocking density with the pooled data from male and female chickens.

battery cages had a negative effect on broiler performance (Houshmand et al., 2012; Zhang et al., 2013). The variation in the upper limits of high stocking density among previous experiments may be a consequence of the differences in the experimental design such as environmental conditions, feeding periods, and gradations of stocking densities (Buijs et al., 2009).

For the effects of sex on growth performance, male broiler chickens had a greater FE than female broiler chickens. Similar improvements in FE were observed in previous experiments (Lippens et al., 2000; Zuowei et al., 2011). In addition, male broiler chickens had a greater CV of the final BW (i.e., less uniformity) than female broiler chickens in the current experiment, which accorded with Peak et al. (2000) who observed that male broiler chickens had less uniformity than female broiler chickens. No interaction between sex and stocking density was observed in this experiment, which indicates that the difference in growth performance due to sex was independent of stocking density. This result agreed with Madilindi et al. (2018) who observed a similar negative effect of increasing stocking density on growth performance between sexes. However, Zuowei et al. (2011) reported that growth depression in female chickens was more observable by high stocking density than male chickens at the marketing age. Variation in BW at different stocking densities and environmental conditions among experiments may be responsible for these inconsistent results.

As observed in previous experiments (Havenstein et al., 1994; Corzo et al., 2005; Zuowei et al., 2011), female broiler chickens had a greater breast meat yield compared with male broiler chickens. Corzo et al. (2005) reported that female broiler chickens had a larger proportion of breast, tender, and abdominal fat than male broiler chickens, whereas male broiler chickens had a larger proportion of drumsticks and saddle than female broiler chickens. Therefore, inherent differences in carcass characteristics between sexes may be the reason for the different breast meat yield. The observation that high stocking density had no effects on breast meat quality in the current experiment agreed with previous experiments (Bilgili and Hess, 1995; Feddes et al., 2002; Zuowei et al., 2011).

Impairments in intestinal barrier function have been considered the potential reason for decreased productive performance and increased incidences of health problems for animals (Lambert, 2009; Hu et al., 2013). Therefore, the effects of various nutritional and environmental factors on intestinal barrier function in animals have been determined, mainly based on intestinal permeability measured by either TER values using the Ussing chamber (Pearce et al., 2013; Song et al., 2014) or LPS concentrations in the blood (Lambert, 2009) as

**Table 4.** Effects of stocking density and sex on intestinal permeability of broiler chickens.

| Item | | Intestinal barrier permeability[1] | | | |
|---|---|---|---|---|---|
| | | PD, mV | Isc, $\mu$a/cm$^2$ | TER, $\Omega$/cm$^2$ | LPS, EU/ml |
| Sex | SD[2] | | | | |
| Male | 15.2 | 124 | 0.36 | 345 | 25.7$^{c}$ |
| | 20.2 | 145 | 0.36 | 447 | 27.2$^{c}$ |
| | 25.3 | 104 | 0.42 | 274 | 38.3$^{b,c}$ |
| | 30.4 | 83 | 0.42 | 214 | 94.7$^{a}$ |
| Female | 15.2 | 161 | 0.38 | 451 | 35.3$^{b,c}$ |
| | 20.2 | 187 | 0.48 | 450 | 46.6$^{b,c}$ |
| | 25.3 | 140 | 0.32 | 420 | 52.9$^{b}$ |
| | 30.4 | 119 | 0.75 | 227 | 39.1$^{b,c}$ |
| SEM (n = 5) | | 32.3 | 0.133 | 64.4 | 8.42 |
| Main effect | | | | | |
| Sex | | | | | |
| Male | | 114 | 0.39 | 320 | 46.5 |
| Female | | 152 | 0.48 | 387 | 43.5 |
| SEM (n = 20) | | 16.2 | 0.067 | 32.2 | 4.21 |
| SD | | | | | |
| 15.2 | | 143 | 0.37 | 398$^{a}$ | 30.5$^{b}$ |
| 20.2 | | 166 | 0.42 | 449$^{a}$ | 36.9$^{b}$ |
| 25.3 | | 122 | 0.37 | 347$^{a,b}$ | 45.6$^{b}$ |
| 30.4 | | 101 | 0.58 | 221$^{b}$ | 66.9$^{a}$ |
| SEM (n = 10) | | 22.9 | 0.094 | 45.6 | 5.96 |
| Effect ($P$-value) | df | | | | |
| Sex | 1 | 0.108 | 0.339 | 0.151 | 0.614 |
| SD | 3 | 0.238 | 0.337 | 0.009 | <0.001 |
| Sex × SD | 3 | 0.999 | 0.438 | 0.626 | <0.001 |
| SD (linear)[3] | 1 | 0.108 | 0.171 | 0.004 | <0.001 |
| SD (quadratic)[3] | 1 | 0.337 | 0.382 | 0.061 | 0.221 |

[a–c]Means in the same column with different superscripts are different ($P < 0.05$).

[1]PD, trans-epithelial voltage; Isc, short circuit current; TER, trans-epithelial electrical resistance; LPS, serum lipopolysaccharide (EU, endotoxin units/ml).

[2]SD, stocking density (birds/m$^2$).

[3]Linear and quadratic effects of increasing stocking density with the pooled data from male and female chickens.

**Table 5.** Effects of stocking density and sex on tight junction-related gene expression in the jejunal mucosa of broiler chickens.

| Item | | Gene expression[1] | | | |
|---|---|---|---|---|---|
| | | *ZO-1* | *OCLN* | *CLDN-1* | *JAM-2* |
| Sex | SD[2] | | | | |
| Male | 15.2 | 2.31 | 1.08 | 1.07 | 1.72 |
| | 20.2 | 1.34 | 1.01 | 1.16 | 1.00 |
| | 25.3 | 1.09 | 1.11 | 0.83 | 0.99 |
| | 30.4 | 0.97 | 0.99 | 0.76 | 0.70 |
| Female | 15.2 | 1.04 | 1.39 | 0.74 | 0.86 |
| | 20.2 | 1.15 | 1.18 | 0.99 | 1.02 |
| | 25.3 | 0.87 | 1.42 | 1.01 | 0.77 |
| | 30.4 | 0.67 | 0.69 | 0.80 | 0.55 |
| SEM (n = 5) | | 0.416 | 0.328 | 0.237 | 0.205 |
| Main effect | | | | | |
| Sex | | | | | |
| Male | | 1.43 | 1.05 | 0.96 | 1.10 |
| Female | | 0.93 | 1.17 | 0.88 | 0.80 |
| SEM (n = 20) | | 0.208 | 0.164 | 0.126 | 0.102 |
| SD | | | | | |
| 15.2 | | 1.67 | 1.23 | 0.90 | 1.29$^{a}$ |
| 20.2 | | 1.25 | 1.10 | 1.08 | 1.01$^{a,b}$ |
| 25.3 | | 0.98 | 1.26 | 0.92 | 0.88$^{a,b}$ |
| 30.4 | | 0.82 | 0.84 | 0.78 | 0.63$^{b}$ |
| SEM (n = 10) | | 0.294 | 0.232 | 0.168 | 0.145 |
| Effect ($P$-value) | df | | | | |
| Sex | 1 | 0.112 | 0.610 | 0.684 | 0.053 |
| SD | 3 | 0.226 | 0.605 | 0.678 | 0.030 |
| Sex × SD | 3 | 0.535 | 0.786 | 0.737 | 0.171 |
| SD (linear)[3] | 1 | 0.045 | 0.350 | 0.496 | 0.004 |
| SD (quadratic)[3] | 1 | 0.670 | 0.559 | 0.362 | 0.942 |

[a,b]Means in the same column with different superscripts are different ($P < 0.05$).

[1]*ZO-1*, zonula occludens-1; *OCLN*, occludin; *CLDN-1*, claudin-1; *JAM-2*, junctional adhesion molecule B.

[2]SD, stocking density (birds/m$^2$).

[3]Linear and quadratic effects of increasing stocking density with the pooled data from male and female chickens.

well as selective tight junction-related gene and protein expression in the GIT (Zhang and Guo, 2009; Shin et al., 2018).

The decreased TER values and increased serum LPS concentrations by increasing stocking density in broiler chickens were observed in the current experiment. Therefore, our observation indicates that high stocking density can increase intestinal permeability as an indicative of decreased intestinal barrier function. This is likely one of reasons why high stocking density decreased growth performance of broiler chickens in the current experiment. Decreased intestinal barrier function by high stocking density appears to be related to elevated stress of broiler chickens. Lambert (2009) suggested that various stressful conditions including psychological stress, severe exercise, and heat stress result in decreased intestinal blood flow, which damages intestinal barrier functions. Interestingly, although there was a linear decrease in TER values but a linear increase in serum LPS concentrations with increasing stocking density, stocking density of less than 25.3 birds/m$^2$ had no negative effects on TER values and serum LPS concentrations. This result accorded with the observation for growth performance in this experiment. As a result, it can be suggested that the negative effect of stocking density in broiler chickens is highly associated with impaired intestinal barrier function.

No differences in TER values were observed between male and female chickens, regardless of stocking density. However, there was an interaction for serum LPS concentrations between sex and stocking density because increasing stocking density did not affect serum LPS concentrations in female broiler chickens, but increased serum LPS concentrations in male broiler chickens with male broiler chickens raised at stocking density of 30.4 birds/m$^2$ showing the greatest LPS concentrations. This result is difficult to be explained because there was no interaction between sex and stocking density for TER values, which are highly correlated with serum LPS concentrations as observed in the current experiment.

Intestinal barrier function is often determined by tight junction integrity of epithelial layers because tight junction plays an important role in sealing the apical gaps of enterocytes in the intestinal epithelium (Moretó and Pérez-Bosque, 2009). The decreased expression of tight junction-related genes and proteins has been considered a molecular evidence for impaired intestinal barrier functions (Moretó and Pérez-Bosque, 2009; Gilani et al., 2017). Therefore, we measured selected tight junction-related gene expression in the jejunal

mucosa. The *OCLN*, *CLDN-1*, and *JAM-2* are transmembrane proteins of the tight junction, which are associated with maintaining the integrity of the tight junction and regulating its barrier function (Ulluwishewa et al., 2011). The *ZO-1* is an important scaffolding protein connecting transmembrane proteins to intracellular cytoskeletons (Ulluwishewa et al., 2011).

There were no interactions between stocking density and sex, and no main effects of sex on those selected gene expression was observed. This result may indicate that tight junction-related gene expression is not different between sexes, and furthermore, the effects of sex were similar, irrespective of stocking density. However, a linear decrease in the expression of *ZO-1* and *JAM-2* was observed as stocking density was increased in this experiment, which demonstrated that increasing stocking density decreased the integrity of tight junction in the jejunal mucosa. This result also was coincident with our observation for decreased TER values and increased serum LPS concentrations by high stocking density. On the other hand, the expression of *OCLN* and *CLDN-1* was not influenced by stocking density although those genes also are known to play a role in tight junction integrity. Likewise, Shin et al. (2018) reported that dietary supplementation of betaine selectively modified jejunal tight junction-related gene expression in laying hens exposed to heat stress. The possible reason for these selective changes in tight junction-related gene expression is not clear because of limited data pertaining to the association between intestinal barrier function and tight junction-related gene expression in animals. Therefore, further molecular studies are required to elucidate the effects of stocking density and sex on tight junction-related gene expression in broiler chickens and their association with intestinal barrier function.

## CONCLUSIONS

Increasing stocking density decreases BWG of broiler chickens raised in battery cages, which is potentially due to a decreased FI with increasing stocking density. In addition, the decreased BWG is likely associated with impaired intestinal barrier function characterized by increased intestinal permeability and selective modification of intestinal tight junction-related gene expression. Therefore, dietary regimens and managements to improve intestinal barrier function may aid in ameliorating the adverse effect of high stocking density on growth performance and health of broiler chickens.

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