

# Effects of nicotinamide and sodium butyrate on meat quality and muscle ubiquitination degradation genes in broilers reared at a high stocking density

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**ABSTRACT** This study was conducted to investigate the effects of supplementation with nicotinamide (NAM) and sodium butyrate (BA) on meat quality and expression of muscle development genes in broilers reared at a high stocking density. A total of 567, 21-day-old AA broilers were randomly assigned to 5 treatment groups and 2 control groups, with 7 replicates of each group. The control groups included a low stocking density (LD; 12.9 birds/m<sup>2</sup>) and were fed a basal diet. The treatment groups were kept at a high stocking density (HD; 18.6 birds/m<sup>2</sup>) and received either a low dose of NAM (50 mg/kg; treatment LN), a high dose of NAM (100 mg/kg; treatment HN), a low dose of BA (500 mg/kg; treatment LB), a high dose of BA (1,000 mg/kg; treatment HB), or a compound supplement (50 mg/kg NAM+500 mg/kg BA; treatment COMB); broilers were reared till 42 D of age. The control groups were kept at HD or at LD (12.9 birds/m<sup>2</sup>) and were fed a basal diet. The heterophil-to-lymphocyte

ratio was significantly higher in the HD control group than that in the LD group; this ratio was significantly lower in treatments LN, HN, HB, and COMB than that in the HD control group. The lightness of breast muscles at 45 min and 24 h after slaughter was significantly higher in the HD group than that in the LD group, and the HD group showed the highest drip loss at 24 h and 48 h. Lightness and drip loss were lower in the HN, LB, and COMB treatments than those in the HD group. HD rearing significantly reduced gene expression of myogenic regulatory factor 5 (*MYF5*) while significantly increased expression of the protein ubiquitin degradation genes *FBXO9*, *FBXO22*, and *FBXO32*. All treatments significantly reduced *FBXO9* and *FBXO32* expression. Our results suggest dietary supplementation with NAM and BA can improve meat quality of broilers under high stocking density by upregulating the expression of myogenic genes, and inhibiting protein ubiquitination.

**Key words:** stocking density, broiler, nicotinamide, sodium butyrate, meat quality

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## INTRODUCTION

During the past decades, stocking density in poultry breeding has dramatically increased to improve profitability (Kiani and von Borstel, 2019). However, high stocking density has adverse effects on production performance and animal welfare in broilers (Averos and Estevez, 2018; Goo et al., 2019). In a previous study, we found that high stocking density reduced the

pectoral muscle's total antioxidant capacity (T-AOC) and reduced expression of antioxidant proteins such as regucalcin and catalase in the liver (Wu et al., 2018). Furthermore, high stocking density has been shown to cause oxidative stress in broilers (Najafi et al., 2015), and oxidative stress is associated with high drip loss and lowered pH of broiler breast muscle (Chen et al., 2010; Wang et al., 2017). Moreover, high stocking density also affects meat quality in terms of reduced tenderness (Patria et al., 2016; Zhang et al., 2018).

Oxidative stress can overactivate the ubiquitin-proteasome system, which causes protein degradation and skeletal muscle atrophy (Abrigo et al., 2018). The F-box proteins (*FBXO*) are crucial for the process of ubiquitin-dependent proteolysis in eukaryotes (Yoshida, 2007). *FBXO32*, a member of the F-box protein family, is a commonly used marker of accelerated

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proteolysis and atrophy processes (Cohen et al., 2015). Thus, *FBXO32* expression is considered as a suitable marker for assessing muscle mass in broiler chickens for breeding selection (Nakashima et al., 2009).

Nicotinamide (NAM) can upregulate the expression of antioxidant genes (John et al., 2012) and improve stress resistance ability (Tran et al., 2016). In addition, NAM reduces the formation of carbonylated proteins in the liver of high-fat-fed mice (Mitchell et al., 2018). Dietary supplementation with high doses of NAM (150 mg/kg) can increase the content of unsaturated fatty acids in breast muscle of broilers reared at high stocking densities (Piva et al., 1993). Furthermore, supplementation with NAM can improve muscle quality, and dietary supplementation with 60 mg/kg niacin (the precursor of NAM) reduced drip loss in broiler breast muscle (Jiang et al., 2011).

Sodium butyrate (BA) was shown to improve meat quality in broilers subjected to experimental corticosterone injections (Zhang et al., 2011) and to affect muscle structure by increasing the ratio of slow-type muscle fibers in the quadriceps of mice (Henagan et al., 2015). In a previous study, we identified a synergistic effect of NAM and BA on growth performance and mitochondrial function in broilers (Wu et al., 2019). However, the individual contribution of each of these 2 compounds to meat quality in broilers has not yet been investigated.

Therefore, this study was conducted to assess the effect of supplementation with NAM and BA on meat quality and to investigate the expression of putative genes associated with meat quality.

## MATERIAL AND METHODS

### Ethics Statement

All procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of the China Agricultural University, Beijing, China.

### Experimental Animals and Grouping

A total of 567, 21-day-old AA broilers were randomly assigned to 7 groups, which included 2 control groups and 5 treatment groups, using 7 replicates of each group.

The control groups were kept at a low stocking density (LD; 12.9 birds/m<sup>2</sup>; 9 birds per cage) or high stocking density (HD; 18.6 birds/m<sup>2</sup>; 12 birds per cage) and were fed a basal diet. Stocking densities used in this study are based on the study of Vargas-Galicia et al. (2017). The treatment groups were kept at HD, and their diet was supplemented with a low dose of NAM (50 mg/kg; treatment LN), a high dose of NAM (100 mg/kg; treatment HN), a low dose of BA (500 mg/kg; treatment LB), high dose of BA (1,000 mg/kg; treatment HB), or compound addition (50 mg/kg NAM plus 500 mg/kg BA; treatment COMB); broilers were reared to an age of 42 D.

The feed additives NAM (99%; Jiangxi Brothers Medicine Co. Ltd., Jiujiang, China) and BA (30%,

encapsulated; Hangzhou King Technology Feed Co. Ltd., Hangzhou, China) were purchased from a commercial distributor.

### Experimental Diets

The composition and nutrient levels of basal diet are shown in Table 1. The diets were formulated to meet or exceed minimum nutrient requirements as recommended by the National Research Council (1994).

### Raising Management

The experiment was conducted on the experimental chicken farm of the College of Animal Science and Technology, China Agricultural University, Beijing, China. Broilers were raised from 21 to 42 D of age on a plastic wire floor. The respective diet and clean drinking water were continuously provided *ad libitum*. Ambient temperature was kept at 20–21°C, and the illumination period was 18 h per day. The animals' health status was monitored daily.

### Data Collection and Sampling

On day 42 and after 5 h of starvation, the broilers' body weight (BW) and the remaining feed per cage were weighed. Feed intake (FI) and body weight gain (BWG) were determined to calculate the feed conversion rate (FCR).

One bird per cage was randomly selected to collect serum and breast muscle samples. A 4-mL blood sample was collected from the wing vein of one broiler per replicate and was placed in a coagulation tube. After centrifugation at 3,000 × *g* and 4°C for 10 minutes, serum was separated and stored at –20°C. Blood samples of 2 mL were collected using anticoagulation tubes to conduct

**Table 1.** The composition and nutrient level of basal diet.

Ingredient	%	Nutrient parameters	Nutrient level (%)
Corn	60.73	AMEn	3,110 kcal/kg
Soybean meal	22.70	Crude protein	19.05
Corn gluten meal	6.00	Lysine	1.12
Wheat bran	2.00	Methionine	0.53
Soybean oil	4.10	Threonine	0.71
DL- Methionine	0.22	Tryptophan	0.19
L-Lysine sulphate	0.60	Calcium	0.93
Threonine	0.03	Available phosphorus	0.41
Sodium chloride	0.30	Met + Cys <sup>3</sup>	0.80
Choline chloride (50%)	0.20		
Trace mineral premix <sup>1</sup>	0.20		
Vitamin premix <sup>2</sup>	0.02		
Dicalcium phosphate	1.70		
Limestone	1.20		

<sup>1</sup>The trace mineral premix provided the following per kg of diets: Cu, 16 mg (as CuSO<sub>4</sub>·5H<sub>2</sub>O); Zn, 110 mg (as ZnSO<sub>4</sub>); Fe, 80 mg (as FeSO<sub>4</sub>·H<sub>2</sub>O); Mn, 120 mg (as MnO); Se, 0.3 mg (as Na<sub>2</sub>SeO<sub>3</sub>); I, 1.5 mg (as KI); Co, 0.5 mg.

<sup>2</sup>The vitamin premix provided the following per kg of diets: vitamin A, 10,000 IU; vitamin D3, 2,400 IU; vitamin E, 20 mg; vitamin K3, 2 mg; vitamin B1, 2 mg; vitamin B2, 6.4 mg; VB6, 3 mg; VB12, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; pantothenic acid, 10 mg; nicotinamide, 30 mg.

<sup>3</sup>Met + Cys: methionine + cysteine.

**Table 2.** Primer sequences of real time PCR.

Gene	Primer sequence (5'-3')	Size	Accession no.
<i>Beta-actin</i>	Forward: CAACACAGTGTCTGCTGGTGGTAC Reverse: CTCCTGCTTGTGATCCACATCTG	199	L08165.1
<i>MSTN</i>	Forward: AGTGGCTCTGGATGGCAGTAGTC Reverse: TCTGTCTCCACGTACAAGCATTC	80	NM_001001461.1
<i>FBXO32</i>	Forward: GCCAGTACCACTTCACAGACAGAC Reverse: GCGTGTCCACATACTGCTCCTTC	132	NM_001030956.1
<i>MYF5</i>	Forward: GCGGAAGGCAGCCACTATGAG Reverse: CGATGTACCTGATGGCGTTCCTC	143	NM_001030363.1
<i>MYOG</i>	Forward: AGCAGGAGCGTGAGCAGAGG Reverse: CGATGGAGGAGAGCGAGTGGAG	198	NM_204184.1
<i>MYF6</i>	Forward: CTGCTGCACAGGCTGGATCAG Reverse: AGGCCGACGACTCCACCATG	199	NM_001030746.1
<i>TNNI1</i>	Forward: AGAAGGAGGACACGGAGAAGGAG Reverse: TTGGCGGCGTCCAACATCTTC	110	XM_004934839.3
<i>TNNI2</i>	Forward: TGGAGGACCTGAGCCAGAAGC Reverse: ACGCAGCATGGCATCAGCAG	95	NM_205417.1
<i>FBXO7</i>	Forward: AGTGCCACTGATGCCTTGATTGTC Reverse: GCATAGACACTGCCTTGGCTTCTG	94	NM_001012537.2
<i>FBXO8</i>	Forward: CAGCAACTGCAACACCAAGGATTC Reverse: TTCCGGTGGCTTGTATTGGAGAC	101	NM_001006446.1
<i>FBXO9</i>	Forward: CTATAGAGCGTGGCACCAAGTGG Reverse: TGTGGATCTTCAGGCGTTGTAAGC	93	NM_001006414.1
<i>FBXO22</i>	Forward: CCAGAGTGCCACGGTTTTGT Reverse: CTCGGGGATGTTTGCTGCTT	94	NM_001030545.1
<i>FBXO34</i>	Forward: TGGCTACGGAGCGAGGTGTG Reverse: CAGCGTGAGCAAGTCCTGAACC	122	NM_001031213.1
<i>FBXO38</i>	Forward: AGTATGGCTTGGCTGATGTGGTTG Reverse: CATCGCACCAATGTGAGGTCTACC	190	JX290204.1
<i>VEGF</i>	Forward: CGGAGTTGTCTGAAGGCTGCTC Reverse: CGCACATCTCATCAGAGGCACAC	191	AB011078.1
<i>ANGPT1</i>	Forward: GGATGGTGGTTCGATGCCTGTG Reverse: GTGGTGGAACGCAAGGAGTAGC	134	NM_001199447.3
<i>ANGPT2</i>	Forward: TCTGGACTCACAACAAGTGAACC Reverse: GCTGCCACCTTCACGTCTCTG	129	NM_204817.1
<i>ANGPTL2</i>	Forward: GCTGCTGTAGGAAGTCTGCTAG Reverse: CCTGTGGATCACTGGAACGCTTG	112	NM_001277699.1
<i>ANGPTL3</i>	Forward: ACGTTACGTCGAGTATGCGTTCAC Reverse: CCATGTCACGGTCCGAGTTG	140	NM_001135122.1

**Table 3.** Effect of NAM and BA supplementation and stocking density on production performance of broilers from 21 to 42 D old.

Items	LD	HD	LN	HN	LB	HB	COMB	SEM	P value
BW/g	2,448	2,285	2,256	2,274	2,282	2,251	2,282	21.6	0.204
BWG/g	1,517	1,358	1,326	1,356	1,340	1,333	1,361	21.2	0.210
FI/g	2,691	2,536	2,496	2,534	2,483	2,495	2,556	23.8	0.267
FCR	1.78	1.87	1.89	1.88	1.85	1.88	1.89	0.01	0.299

Data are presented as mean  $\pm$  SEM.

BA, sodium butyrate; COMB, compound addition 50 mg/kg NAM plus 500 mg/kg BA + HD; LB, low dose of BA (500 mg/kg); LD, low stocking density (12.9 birds/m<sup>2</sup>) group; LN, low dose of NAM (50 mg/kg) + HD; HB, high dose of BA (1,000 mg/kg); HD, high stocking density group (18.6 birds/m<sup>2</sup>) group; HN, high dose of NAM (100 mg/kg) + HD; NAM, nicotinamide; 1, SEM, standard error of mean.

routine blood tests. The broilers were killed by cervical dislocation after blood collection.

Immediately after slaughter, the breast muscle on the animal's right side was removed for meat quality evaluation.

### Meat Quality Evaluation

To determine drip loss, approximately 10 g of muscle was weighed (W1) and placed in a sealed polyethylene bag at for storage 4°C. The muscle was reweighed (W2) after 24 h, and drip loss was calculated as (W1–W2)/W2  $\times$  100%.

Cooking loss was determined according to the method suggested in the study by Cai et al. (2018) and was calculated according to the the following formula: (initial weight – final weight)/initial weight  $\times$  100%.

At 45 min and 24 h postmortem, meat color was measured using an automatic Chroma meter (3nh NR-10QC; Shenzhen Three nh Technology Co., Ltd., Shenzhen, China) set to the color system of lightness (L\*), redness (a\*), and yellowness (b\*). Each sample was measured 3 times at 3 different locations (cranial, medial, and caudal portion) on the medial surface of each fillet.

The pH of breast muscle was measured at 45 min, 24 h, and 48 h after slaughter using a pH meter (Testo 205; Testo, Berlin, Germany), and pH of leg muscle was measured for 45 min and 24 h after slaughter.

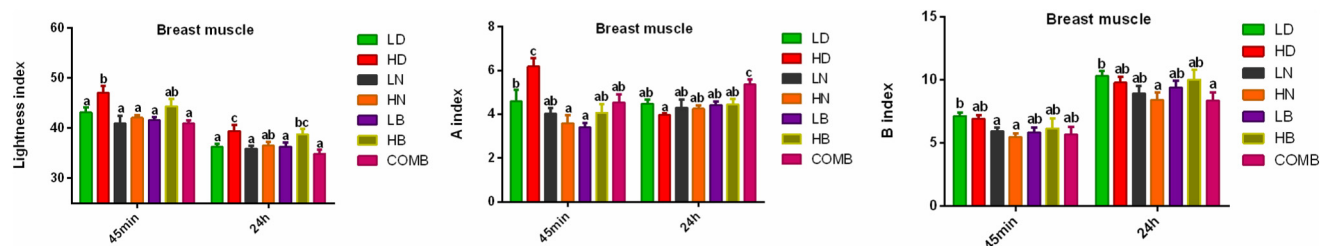
### Enzyme Activity Determination in Serum

Blood routine tests were performed by a conductivity method using a blood biochemical analyzer (XS-500i; Sysmex Co., Kobe, Japan).

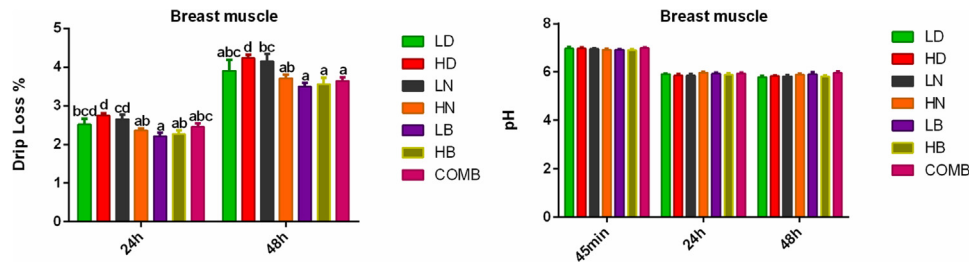
T-AOC (cat. # A015); enzymatic activities of superoxide dismutase (cat. # A001-3), lactate dehydrogenase (cat. # A020-2), creatine kinase (cat. # A032), and xanthine oxidase (XOD; cat. # A002-1); and content of malondialdehyde (cat. # A003-1) in serum were determined according to the instructions of the Jian Cheng Bioengineering Institute (Nanjing, China).

### Expression of Muscle Development Genes

Total RNA of broiler muscle samples was extracted using Trizol reagent (TAKARA BIO Inc., Kusatsu, Japan), and RNA purity and concentration were measured using a nucleic acid analyzer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA). The cDNA was produced using a reverse transcription kit (PrimeScript RT Reagent Kit with gDNA Eraser [Perfect Real Time]; cat. # RR047 A; TAKARA BIO Inc., Kusatsu, Japan) and was stored at –80°C for further analysis. Fluorescence quantitative PCR was performed using a TB Green™ Premix Ex Taq™ kit (Tli RNaseH Plus; cat. # RR420 A; TAKARA BIO Inc., Kusatsu, Japan) according to the manufacturer's instructions. The detection device was a 7500-fluorescence detection system (Applied Biosystems, Foster City, CA). The PCR reaction conditions were as follows: initial denaturation at 95°C for 30 s was followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. After PCR amplification, a dissolution curve was produced, and agarose gel electrophoresis was performed to confirm the respective fragment size. PCR primer sequences for the target gene fragments and the internal reference gene (beta-actin) are shown in table 2. Gene expression values were calculated using the 2<sup>– $\Delta\Delta$ CT</sup> method.



**Figure 1.** Meat color of breast muscle at 45 min and 24 h Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ). Data are presented as mean  $\pm$  SEM. SEM, standard error of mean.



**Figure 2.** Drip loss and pH of breast muscle. Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ). Data are presented as mean  $\pm$  SEM. SEM, standard error of mean.

## Statistical Analysis

Results were shown as means and standard errors of the mean. A one-way ANOVA was applied for single-factor analysis using SPSS 20.0 (SPSS Inc. Chicago, IL). Statistical significance is reported at  $P < 0.05$ . Trends are reported at  $P < 0.1$ .

## RESULTS

### Performance and Meat Quality

Stocking density had no significant effect on FI, BW, BWG, and FCR, and supplementation with NAM or BA produced no significant effect on broiler production performance (Table 3).

However, stocking density significantly affected breast muscle lightness at 45 min and 24 h, with higher values in the HD group than in the LD group ( $P < 0.05$ ). Supplementation with BA or NAM significantly decreased breast muscle lightness at 45 min and 24 h after slaughter, compared with the control, apart from the HB treatment ( $P < 0.05$ ) (Figure 1).

Breast muscle redness at 45 min after slaughter was significantly higher in the HD group than in the LD group ( $P < 0.05$ ) (Figure 1) but was lower in the HD group than in the LD group after 24 h. At 45 min, redness values of the LN, HN, LB, and COMB groups were significantly lower than that of the HD group ( $P < 0.05$ ), and at 24 h, redness of COMB was significantly higher than that of the HD group ( $P < 0.05$ ). No significant difference was observed between groups HD and LD.

There was a significant difference in the drip loss of breast muscle between groups ( $P < 0.05$ ), and the highest drip loss was observed in the HD group at 24 h and 48 h. The drip loss in HN, LB, HB, and COMB group was lower than that in the HD group at 24 h and 48 h ( $P < 0.05$ ) (Figure 2).

Stocking density had no significant effect on the pH of the breast muscle at 45 min, 24 h, and 48 h (Figure 2).

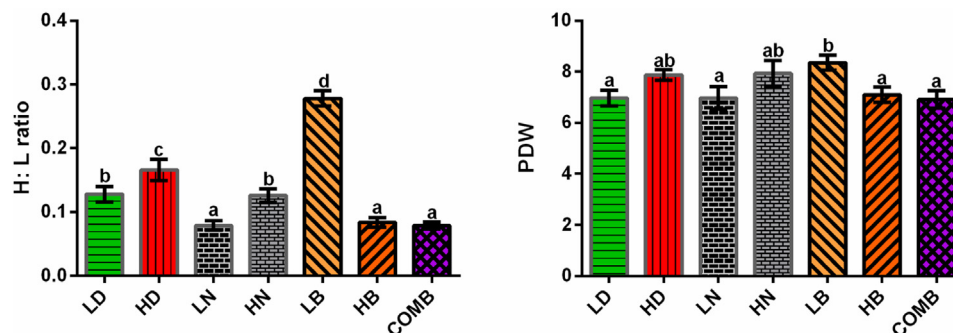
### Blood Indices

The heterophil-to-lymphocyte ratio was significantly higher in the HD group than that in the LD group ( $P < 0.05$ ). In the LN, HN, HB, and COMB treatments, this ratio was significantly lower than that in the HD group ( $P < 0.05$ ). Platelet distribution width showed a similar pattern as the heterophil-to-lymphocyte ratio (Figure 3).

Stocking density had no significant effect on serum superoxide dismutase activity or T-AOC. Similarly, stocking density had no significant effect on oxidative stress markers, that is, creatine kinase and lactate dehydrogenase, and neither on malondialdehyde or XOD. However, serum XOD activity was significantly lower in the LN treatment than that in the HD group ( $P < 0.05$ ) (Table 4).

### Expression of Muscle Development Genes

Gene expression of myogenic regulatory factor 5 (*MYF5*) was significantly reduced in the HD group, compared with the LD group. The expression of *MYF5*



**Figure 3.** The blood heterophil-to-lymphocyte (H:L) ratio and platelet distribution width (PDW) of broilers. Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ). Data are presented as mean  $\pm$  SEM. SEM, standard error of mean.

**Table 4.** Effect of NAM and BA supplementation and stocking density on serum antioxidant and oxidative stress indicators.

Items	LD	HD	LN	HN	LB	HB	COMB	SEM	P value
SOD/U/mL	68.74	70.15	79.43	69.28	78.95	78.78	70.05	1.69	0.148
T-AOC/U/mL	1.68	1.53	1.81	1.73	1.60	1.74	1.82	0.05	0.823
CK/U/mL	3.23 <sup>a,b</sup>	3.36 <sup>a,b,c</sup>	3.62 <sup>b,c,d</sup>	3.93 <sup>c,d</sup>	4.04 <sup>d</sup>	3.94 <sup>c,d</sup>	2.94 <sup>a</sup>	0.09	0.001
LDH/U/L	7,542.37 <sup>a,b</sup>	7,244.55 <sup>a,b</sup>	8,916.01 <sup>b,c</sup>	8,324.88 <sup>b,c</sup>	9,495.72 <sup>c</sup>	9,128.37 <sup>b,c</sup>	7,449.33 <sup>a,b</sup>	239.72	0.006
MDA/nmol/mL	0.25	0.23	0.19	0.18	0.24	0.42	0.35	0.03	0.136
XOD/U/L	0.85 <sup>b,c,d</sup>	0.84 <sup>b,c,d</sup>	0.30 <sup>a</sup>	0.93 <sup>c,d</sup>	0.59 <sup>a,b,c</sup>	0.29 <sup>a,b</sup>	1.52 <sup>d,e</sup>	0.07	<0.001

Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ).

Data are presented as mean  $\pm$  SEM.

BA, sodium butyrate; CK, creatine kinase; COMB, compound addition 50 mg/kg NAM plus 500 mg/kg BA + HD; LB, low dose of BA (500 mg/kg); LD, low stocking density (12.9 birds/m<sup>2</sup>) group; LDH, lactate dehydrogenase; LN, low dose of NAM (50 mg/kg) + HD; HB, high dose of BA (1,000 mg/kg); HD, high stocking density group (18.6 birds/m<sup>2</sup>) group; HN, high dose of NAM (100 mg/kg) + HD; MDA, the content of malondialdehyde; NAM, nicotinamide; SEM, standard error of mean; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; XOD, xanthine oxidase.

was significantly higher in the LB treatment than that in the HD group ( $P < 0.05$ ). Stocking density had no significant effect on the expression of myogenic regulatory factor 6 (*MYF6*), myogenin (*MYOG*), and myostatin (*MSTN*). Relative gene expression of *MYOG* was higher in treatments LB and COMB than in HD group ( $P < 0.05$ ). In contrast, gene expression of *MSTN* was significantly lower in treatments LN, HN, and HB groups than that in the HD group ( $P < 0.05$ ) (Table 5).

Compared with the LD treatment, the HD group showed a significant increase in gene expression of *FBXO9*, *FBXO22*, and *FBXO32*. Expression levels of *FBXO9* and *FBXO32* were significantly lower in all treatment groups than those in the HD group ( $P < 0.05$ ), and expression levels of *FBXO22* and *FBXO38* were significantly lower in LN, HN, LB, and HB treatments than in the HD group ( $P < 0.05$ ) (Table 6).

Stocking density had no significant effect on gene expression of troponin I type I (*TNNI1*) and troponin I type 2 (*TNNI2*) genes. Noticeably, the *TNNI1:TNNI2* ratio was significantly higher in the LN treatment than in the HD group ( $P < 0.05$ ). Gene expression of the vascular endothelial growth factor (*VEGF*) was significantly higher in the HD group than in the LD group ( $P < 0.05$ ). Expression levels of *VEGF* were significantly lower in the HN, LB, and HB treatments than in the HD group ( $P < 0.05$ ). Stocking density had no significant effect on expression of *ANGPT1*, *ANGPT2*, *ANGPTL2*, and *ANGPTL3* ( $P > 0.05$ ); however, *ANGPTL2* expression was significantly higher ( $P < 0.05$ ) in LB and HB than in the HD group (Table 7).

## DISCUSSION

In the present study, stocking density had no significant effect on FI, BW, and FCR of broilers. In line with these results, a previous study found that increasing stocking density from 10 birds/m<sup>2</sup> to 16 birds/m<sup>2</sup> had no adverse effects on BW, BWG, and FCR of caged broilers (Najafi et al., 2015). However, effects of stocking density on growth performance of broilers may depend on the rearing system.

The heterophil-to-lymphocyte ratio is an established tool to measure stress in poultry (Qaid et al., 2016). In the present study, the heterophil-to-lymphocyte ratio was higher in the HD group than that in the LD group, suggesting that high stocking density increases oxidative stress. Supplementation with NAM can upregulate expression of antioxidant genes (John et al., 2012) and can also enhance stress tolerance (Tran et al., 2016). In the present study, the activity of serum XOD was lower in the LN treatment than in the HD group, and inhibition of XOD is known to reduce production of reactive oxygen species (Fernandez et al., 2002). Therefore, dietary supplementation with NAM likely improves antioxidant capacity in broilers. Similarly, BA appears to enhance broiler antioxidant capacity (Wu et al., 2018). In the present study, the heterophil-to-lymphocyte ratio in the LN, HN, HB and COMB treatments was lower than that in the HD group, suggesting that both BA and NAM may help improve resistance against oxidative stress tolerance.

Meat color is one of the most important factors affecting consumer purchase decisions (Mancini and

**Table 5.** Effect of NAM and BA supplementation and stocking density on mRNA expression of muscle development-related genes of broilers from day 21 to 42.

Gene Name	LD	HD	LN	HN	LB	HB	COMB	SEM	P value
MYF5	1.00 <sup>d</sup>	0.53 <sup>b,c</sup>	0.13 <sup>a</sup>	0.31 <sup>a,b</sup>	0.97 <sup>d</sup>	0.74 <sup>c,d</sup>	0.70 <sup>c,d</sup>	0.12	<0.001
MYF6	1.00 <sup>a,b,c</sup>	1.48 <sup>c</sup>	0.88 <sup>a,b,c</sup>	0.37 <sup>a</sup>	0.68 <sup>a,b</sup>	0.94 <sup>a,b,c</sup>	1.25 <sup>b,c</sup>	0.08	0.016
MYOG	1.00 <sup>a,b</sup>	0.86 <sup>a,b</sup>	0.92 <sup>a,b</sup>	0.77 <sup>a</sup>	1.58 <sup>c,d</sup>	1.24 <sup>b,c</sup>	1.77 <sup>d</sup>	0.08	<0.001
MSTN	1.00 <sup>b</sup>	0.98 <sup>b</sup>	0.56 <sup>a</sup>	0.42 <sup>a</sup>	1.21 <sup>b</sup>	0.50 <sup>a</sup>	1.21 <sup>b</sup>	0.07	<0.001

Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ).

Data are presented as mean  $\pm$  SEM.

BA, sodium butyrate; COMB, compound addition 50 mg/kg NAM plus 500 mg/kg BA + HD; LB, low dose of BA (500 mg/kg); LD, low stocking density (12.9 birds/m<sup>2</sup>) group; LN, low dose of NAM (50 mg/kg) + HD; HB, high dose of BA (1,000 mg/kg); HD, high stocking density group (18.6 birds/m<sup>2</sup>) group; HN, high dose of NAM (100 mg/kg) + HD; 1, NAM, nicotinamide; SEM, standard error of mean.

**Table 6.** Effect of NAM and BA supplementation and stocking density on mRNA expression of F-Box Protein Family Genes from day 21 to 42.

Gene Name	LD	HD	LN	HN	LB	HB	COMB	SEM	P value
FBXO7	1.00 <sup>a,b</sup>	1.32 <sup>a,b</sup>	0.77 <sup>a</sup>	0.68 <sup>a</sup>	1.25 <sup>a</sup>	0.95 <sup>a</sup>	1.22 <sup>b</sup>	0.04	<0.001
FBXO8	1.00	1.49	1.23	1.11	1.01	1.15	1.51	0.04	0.078
FBXO9	1.00 <sup>b</sup>	1.71 <sup>c</sup>	1.02 <sup>b</sup>	0.11 <sup>a</sup>	0.71 <sup>b</sup>	0.89 <sup>b</sup>	1.15 <sup>b</sup>	0.05	<0.001
FBXO22	1.00 <sup>a</sup>	1.55 <sup>b</sup>	0.95 <sup>a</sup>	0.81 <sup>a</sup>	0.66 <sup>a</sup>	0.70 <sup>a</sup>	1.08 <sup>a,b</sup>	0.05	0.011
FBXO32	1.00 <sup>b</sup>	2.07 <sup>c</sup>	0.25 <sup>a</sup>	0.30 <sup>a</sup>	0.14 <sup>a</sup>	0.27 <sup>a</sup>	0.36 <sup>a</sup>	0.05	<0.001
FBXO34	1.00	1.40	1.56	1.36	1.08	1.14	1.29	0.05	0.201
FBXO38	1.00 <sup>a,b</sup>	1.21 <sup>b</sup>	0.78 <sup>a</sup>	0.69 <sup>a</sup>	0.72 <sup>a</sup>	0.60 <sup>a</sup>	1.02 <sup>a,b</sup>	0.05	0.019

Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ).

Data are presented as mean  $\pm$  SEM.

BA, sodium butyrate; COMB, compound addition 50 mg/kg NAM plus 500 mg/kg BA + HD; LB, low dose of BA (500 mg/kg); LD, low stocking density (12.9 birds/m<sup>2</sup>) group; LN, low dose of NAM (50 mg/kg) + HD; HB, high dose of BA (1,000 mg/kg); HD, high stocking density group (18.6 birds/m<sup>2</sup>) group; HN, high dose of NAM (100 mg/kg) + HD; 1,NAM, nicotinamide; SEM, standard error of mean.

Hunt, 2005). Our results showed that high stocking density increased the lightness of breast muscle. High meat lightness is typically associated with muscle diseases such as woody meat and pale soft exudative meat (Petracci et al., 2004; Cai et al., 2018). Stress conditions such as heat stress and high CO<sub>2</sub> concentrations have been shown to increase the lightness of breast muscle (Lu et al., 2017; Xu et al., 2018), and high lightness values in the HD group of present study may be due to oxidative stress induced by high stocking density. In the present study, we observed that dietary supplementation with either NAM or BA alone or in combination reversed the increase in lightness of breast muscle, suggesting antioxidant effects NAM and BA.

Stressors such as heat stress can reduce the redness of breast muscle (Wen et al., 2019). In the present study, the HD group showed the lowest redness values at 24 h after slaughter. In addition, our study found the value of 24 h redness in COMB group to be higher than that in the HD group. Generally, low lightness and increased redness values are desired as this indicates high myoglobin concentrations and an improvement in meat color (Kim et al., 2010). Higher redness values also indicate higher oxidative capacity of muscles (Gentry et al., 2004). Dietary supplementation with BA can increase the proportion of type I fibers in skeletal muscle in mice (Henagan et al., 2015), and similarly, supplementation with niacin, a precursor of NAM, increased

the number of oxidative type I fibers in skeletal muscle of growing pigs (Khan et al., 2013) and induced muscle fiber transition from type II to type I in sheep (Khan et al., 2013). The *TNNI1* and *TNNI2* genes were only found in slow and fast fibers, respectively. In this study, stocking density had no significant effect on expression of *TNNI1* or *TNNI2*; however, low doses of NAM significantly increased the *TNNI1*:*TNNI2* ratio. Therefore, higher redness values may be associated with an increase in the ratio of oxidized slow fiber to fast fiber.

Stress conditions such as heat stress can cause increased drip loss in breast muscle (Lu et al., 2017), and high stocking densities increased drip loss in breast muscle of Peking ducks (Zhang et al., 2018). In the present study, the HD group showed the highest drip loss at 24 h and 48 h after slaughter, suggesting that high stocking densities may decrease water-holding capacity in broilers, perhaps because of increased oxidative stress. Previous studies indicated that dietary supplementation with niacin at 60 mg/kg helps reduce drip loss in chicken breast muscle (Jiang et al., 2011) and that supplementation with BA may partially reduce meat quality deterioration (Zhang et al., 2011). In the present study, dietary supplementation with NAM or BA reduced drip loss in breast muscle at 24 h after slaughter. In pork meat, high drip loss seems to be associated with reduced activity of antioxidant enzymes (Chen et al., 2010), and it has been shown that dietary supplementation with organic essential oils or antioxidants such as quercetin can help

**Table 7.** Effect of NAM and BA supplementation and stocking density on mRNA expression of fiber type and vascular development genes from day 21 to 42.

Gene Name	LD	HD	LN	HD	LB	HB	COMB	SEM	P value
TNNI1	1.00	1.04	0.88	0.78	0.79	0.71	1.05	0.05	0.468
TNNI2	1.00	0.85	0.58	0.66	0.63	0.59	0.97	0.06	0.052
TNNI1-2	1.00 <sup>a</sup>	1.17 <sup>a</sup>	1.47 <sup>b</sup>	1.09 <sup>a</sup>	1.19 <sup>a</sup>	1.14 <sup>a</sup>	1.07 <sup>a</sup>	0.03	<0.001
ANGPT1	1.00	0.85	0.91	0.79	0.86	0.68	0.74	0.04	0.314
ANGPT2	1.00	0.90	0.85	0.87	0.89	1.12	0.99	0.03	0.276
ANGPTL2	1.00 <sup>a,b</sup>	0.90 <sup>a,b</sup>	0.70 <sup>a</sup>	0.88 <sup>a,b</sup>	1.39 <sup>c</sup>	1.45 <sup>c</sup>	1.21 <sup>b,c</sup>	0.06	<0.001
ANGPTL3	1.00	1.23	0.95	0.95	1.00	1.01	0.95	0.03	0.221
VEGF	1.00 <sup>a,b</sup>	1.84 <sup>c</sup>	1.92 <sup>c</sup>	0.69 <sup>a</sup>	0.87 <sup>a,b</sup>	0.92 <sup>a,b</sup>	1.45 <sup>b,c</sup>	0.06	0.001

Values with different superscript letters in the same column differ significantly ( $P < 0.05$ ).

Data are presented as mean  $\pm$  SEM.

BA, sodium butyrate; COMB, compound addition 50 mg/kg NAM plus 500 mg/kg BA + HD; LB, low dose of BA (500 mg/kg); LD, low stocking density (12.9 birds/m<sup>2</sup>) group; LN, low dose of NAM (50 mg/kg) + HD; HB, high dose of BA (1,000 mg/kg); HD, high stocking density group (18.6 birds/m<sup>2</sup>) group; HN, high dose of NAM (100 mg/kg) + HD; 1,NAM, nicotinamide; SEM, standard error of mean; TNNI1-2, the TNNI1-to-TNNI2 ratio.

reduce drip loss in meat by enhancing the activity of antioxidant enzymes (Zou et al., 2016). NAM and BA are known to exert antioxidant effects, thus improved water-holding capacity may be associated with higher antioxidant capacity.

Environmental stressors can lead to enhanced muscle proteolysis (Milan et al., 2015), and muscle disorders such as woody meat and striped muscle are often accompanied by severe proteolytic processes (Baldi et al., 2018). The F-box protein family plays a crucial role in protein ubiquitination degradation (Milan et al., 2015), and the protein FBXO32 is a common marker for accelerated proteolysis and atrophy processes (Cohen et al., 2015). Compared with the LD group, HD rearing significantly increased expression of ubiquitin degradation genes such as FBXO9 and FBXO32, which are key genes regarding muscle atrophy. The ubiquitin-proteasome system affects drip loss by regulating the degradation of myofibrils (Milan et al., 2015), and upregulation of the ubiquitination gene UBE3B can increase damage to sarcoplasmic and myofibrillar proteins (Huynh et al., 2013), whereas increased expression of the deubiquitination gene ZRANB1 would enhance the water-holding capacity by promoting protein stability (Ponsuksili et al., 2010; Huynh et al., 2013). The expression levels of FBXO9 and FBXO32 were significantly lower in all treatment groups than those in the HD group. Therefore, supplementation with NAM and BA may improve muscle water-holding capacity by inhibiting the ubiquitination degradation process.

MYF5 is an important transcriptional activator that induces fibroblast differentiation into myoblasts, promotes transcription of muscle-specific target genes, and plays a role in muscle differentiation (Yamamoto et al., 2018). MYOG is essential for muscle homeostasis and for regulating myocyte fusion by determining the number and size of muscle fibers (Ganassi et al., 2018). In the present study, expression of the myogenic regulatory factor gene *MYF5* was significantly reduced in the HD rearing group, compared with that in the LD group. Furthermore, MYOG expression was higher in the treatments LB and COMB than in the HD group. The protein MSTN is a key negative regulator of muscle growth and development, and inhibiting MSTN signaling can increase muscle mass (Shin et al., 2015). In the present study, expression of MSTN was lower in the LN, HN, and HB treatments than in the HD group. These results suggest that high stocking density may impede muscle development by inhibiting the expression of myogenic genes. NAM and BA can accelerate muscle development by elevating the expression of myogenic genes and inhibiting expression of MSTN. VEGF expression was higher in the HD group than in the LD group, as well as with the treatments HN, LB, and HB. Deep chest muscle disease is typically associated with increased VEGFA expression (Yalcin et al., 2018). Thus, HD rearing may increase the risk of muscle disorders; however, dietary supplementation with NAM and BA may help prevent the occurrence of muscle disorder by inhibiting VEGFA expression.

In summary, high stocking density rearing caused abnormal color and reduced water-holding capacity in broiler breast muscle. NAM and BA supplementation may improve meat quality by increasing antioxidants, upregulating the expression of myogenic genes, and inhibiting protein ubiquitination.

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