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Probiotic Supplementation Alleviated Stress and Improved Performance, Meat Quality, Sensory Acceptability and Microbiological Status of Broilers

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ABSTRACT

This study aimed to investigate the efficacy of probiotic supplementation in mitigating stress and improving performance, carcass yield, carcass quality parameters, sensory acceptability, microbiological quality and economic profitability of broilers reared at different stocking densities. Two hundred and forty chicks were divided into four groups; two groups were reared at a low stocking density (LSD, 10 bird/m^2), including one group that was provided with Protexin® probiotic (*Enterococcus faecium*) supplemented in water (LSDP). Similarly, the other two groups were reared at a high stocking density (HSD, 15 bird/m^2), including one group that was provided with the same probiotic supplementation (HSDP). Throughout the growing cycle, bird performance was monitored. At the end of the growing cycle, stress indicators were measured in blood. Besides, carcass and giblet weights, dressing yield, carcass quality parameters, sensory acceptability, microbiological quality and economic profitability were assessed. As a result, HSD mostly impaired broiler performance, increased stress indicators, reduced carcass yields, carcass quality parameters and sensory acceptability, while boosted profitability and slightly lowered microbiological quality. On the other hand, probiotic supplementation reversed the negative effects of HSD. As probiotic supplementation boosted broiler performance, reduced blood stress indicators, increased carcass and giblet yields, carcass quality parameters and sensory scores, besides, it improved the microbiological status of broiler meat in terms of fecal coliforms and *E. coli* MPN. To conclude, rearing broilers at high stocking density induced stress, compromised performance and reduced carcass quality. These negative impacts could be successfully faced by using probiotic supplementation in drinking water.

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Introduction

Global demand for poultry meat has been growing nowadays and chicken became the most famous meat consumed around the world since it has many desirable nutritional values, such as low intramuscular fat content and relatively high concentrations of polyunsaturated fatty acids (Kralik *et al*., 2018). In many countries, the poultry industry is one of the main agricultural industries that considered as the main source of animal protein (Abouelenien *et al*., 2016), that distinguishes from other animal production activities in the relatively high growth rate, feed efficiency utilization (Duclos *et al*., 2007), carcass yield (Meluzzi and Sirri, 2009) and lower costs than ruminants (Beg *et al*., 2011).

Mostly, producers are obliged to rear broilers under high stocking densities in order to reduce the production costs, produce more kilograms of chickens per unit area to meet the increasing demand for a cheap, safe supply of meat and achieve a satisfactory economic return (Abudabos *et al*., 2013; Na-Lampang, 2014; Abouelenien *et al*., 2016). Therefore, high stocking density could decrease market prices of poultry products resulting in additional economic benefits to consumers.

However, high density poses stress on the bird to the extent that the economic profit may come at the cost of reduced bird performance, compromised health, carcass quality and welfare (Dozier *et al*., 2006; Estevez, 2007). Indeed, high stocking densities induce multiple stressors under uncontrolled environmental conditions (Estevez, 2007; Ibrahim *et al*., 2017). These stressors resulted in alterations in the physiological status of birds that reduce the performance and downgrade carcass quality (Xing *et al*., 2019). Thus, facing the stressful effects of high stocking density is an essential target for improving productivity and reducing economic losses.

Direct feed microbials, known as probiotics, are one of the possible approaches employed to avoid physiological stress (Sohail *et al*., 2010; 2011; 2012), improve performance (Huang *et al*., 2004; Cengiz *et al*., 2015), enhance gut microflora ecology (Yu *et al*., 2008), and hence, improving economic efficiency of poultry production. Therefore, this study was conducted to assess the efficacy of probiotic supplementation in mitigating stress and improving the performance, growth rate, carcass quality and muscle microbiota of broilers reared at two differ-

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ent stocking densities in an environmentally uncontrolled conventional house in order to improve bird welfare and economic profitability of broilers production.

Materials and methods

Chemicals and probiotics

Protexin® probiotic [*Enterococcus faecium*, 2×10^9 CFU/g (2×10^{12} CFU/kg)] was purchased from probiotic International Smorest, UK. 30% trichloroacetic acid "TCA, 0.67% of thiobarbituric acid (TBA) and tetramethoxypropane (density of 0.99 g/mL) for measuring malondialdehyde (MDA) were purchased from (E-Mark's, Germany). Corticosterone (CS) kits and chemicals used for interleukin (IL6) analysis were obtained from Sigma Aldrich.

Bird grouping and stocking densities

The study was carried out in the Faculty of Veterinary Medicine, Beni-Suef University, Egypt. A total of 240 unsexed one-day-old chicks (Cobb type breed), purchased from a commercial hatchery at Beni-Suef, were used for this study. The chicks were brooded at 33ºC using electric heaters for the first week of age. Then, at the end of the first week, they were randomly distributed into four groups. Two groups, 48 birds each, were reared at low stocking density (10 birds/m^2) groups, where the first group was provided with a control diet without probiotic supplementation (LSD), while the second group was provided with Protexin® probiotic supplementation in the feed (LSDP). Each group was divided into three replicates (16 bird/ replicate). Whereas the other two groups, 72 birds/ each were reared at high stocking density (15 bird/m^2). The first group was not supplemented with probiotics (HSD), while the other was supplemented with Protexin® probiotic (HSDP). Also, here each group was divided into three replicates (24 bird/replicate). This study design was approved by the Institutional Animal Care and Use Committee of Beni-Suef University (BSU-IACUC), Egypt.

Bird accommodation and probiotic supplementation

Chicks were reared in 12 floor pens of equal dimensions (1 m×1.6 m), with a new clean wood shaving litter material. Proper ventilation was maintained using windows, exhausting fans and fans. The brooding heating was maintained by using electric heaters, with a 2°C decrease in the temperature weekly. A continuous lighting program was used during the first week, then 23 h light / 1 h dark till the end of the experiment. The feed and water were provided adlibtum in well-distributed manual plastic drinkers and feeders. A two-phase broiler-feeding regime was used; a starter diet containing 23% protein crumble during the first 21 days, then a grower pelleted diet with 21% protein till the end of the study at day 42. Protexin® probiotic was added to the drinking water starting from the second week according to the recommended dosage by the manufacturer.

Evaluating the stressful effects of high stocking density and mitigating efficacy of probiotic supplementation

Assessing the performance of birds

Cumulative feed intake (FI), cumulative weight gain (CWG) and cumulative feed conversion ratio (CFCR) were recorded throughout the period from day 8 to day 42 of the growing cycle.

Measuring the stress indicators in blood

Five mL of blood were collected, without anticoagulant for serum separation, from the wing vein of three randomly chosen birds per replicate (9 birds per group), which were fasted overnight. The samples were collected at the end of the growing cycle for the determination of corticosterone, MDA and IL6. After collection, all samples were kept at room temperature for 30 min, and then refrigerated for 15 min before centrifugation at 3000-4000 r.p.m for 10-15 min. Consequently, the serum was aspirated and put in clean Eppendorf tubes and kept frozen at -20ºC until analysis. Serum corticosterone levels were determined using commercial ELISA kits. Lipid peroxidation in the serum was estimated colorimetrically through measuring serum MDA content as described by Albro *et al*. (1986). While IL6 was determined in serum using a real-time PCR according to Suzuki *et al*. (2009).

Carcass quality parameters and yields

All the assessment experiments of carcass quality parameters were done during the 6th week of the growing cycle.

Carcass and giblet yields

At the 6th week of the growing cycle, 2 birds per replicate were fasted overnight and then slaughtered. Afterward, meat samples and organs were collected for the determination of the effect of stocking density and probiotic supplementation on sensory acceptability, carcass quality parameters and the relative and absolute weights of giblets, spleen and bursa. Carcass and giblet yields were calculated according to Beg *et al*. (2011) using the following equations:

Equation (1): Carcass (dressing) yield (g) = Live weight - (blood weight + feathers weight + head weight + shank weight+ digestive system weight)

Equation (2): Dressing % = (Dressing yield / Live weight) × 100

Equation (3): Absolute (g) and relative (%) giblet weight = liver weight + heart weight + gizzard weight + neck weight.

Sensory Acceptability

Sensory evaluation of chicken meat from different groups was conducted according to the method reported by Economou *et al*. (2009). Briefly, pieces of chicken meat (approximately 100 g) from different groups were subjected to cooking in a microwave oven for 20 min. A panel of five well-trained judges was used to evaluate the sensory attributes of chicken meat samples in terms of color, odor, texture and taste. The meat samples were blind coded by special codes and the panelists were not informed about the experimental approach. Panelists were asked to score each sensory attribute using a hedonic scale ranging from 0 to 9. They were informed to wash their mouths with warm water between different samples. The overall acceptability was estimated by calculating the average score of the four attributes; where 9, 8, 7 and 6 represent excellent, very good, good and acceptable grades, respectively. While <6 is poor and unacceptable.

Ultimate pH of Meat

The ultimate pH was determined according to the method recommended by Korkeala *et al*. (1986) in 10 g of muscle added to 10 mL of distilled water using a pH meter.

Drip loss (DL %)

The drip loss was determined according to the method

recommended by Kauffman *et al*. (1986). A slice of muscle (skinless Pectoralis major muscle) of about 2.5 cm thickness and 50-100 g weight was taken from the chicken breast, representing each group. The room temperature during cutting was similar to the meat temperature. The slice of muscle was weighed (W1) and suspended using a net or thread inside a plastic pouch and sealed under atmospheric pressure. The samples were then held at 0-4°C for at least 24 h. The exact duration of storage was reported. The pouches were hanged in such a way that the exudate dripping from the meat does not remain in contact with the meat. At the end of the storage period, the muscle slice was removed from the pouch, dried gently with an absorbing tissue and reweighed (W2). During weighing, care was taken that no condensation of water vapor occurs on the cold surface. Drip loss was calculated from weight loss as follows, drip loss %= [(W1 – W2) /W1] × 100.

Cooking loss (CL %)

Following the drip loss determination, the same sample was used immediately for cooking loss measurement. If there was a delay before cooking loss measurement, the sample would be wrapped to avoid drying out of the surface. Determination of cooking loss was done according to the method of Kauffman *et al*. (1986). Each muscle slice was weighed (W1) and individually placed inside heat resistant and waterproof pouches, and then cooked in a water bath at 80°C until an internal temperature of 70°C was reached. During cooking, the internal temperature was tracked by a portable food thermometer. The cooked samples were then cooled to 4°C, removed from the pouches, gently dried with a filter paper, and reweighed (W2). The cooking loss was calculated according to the following equation: Cooking loss %= [(W1-W2) /W1] ×100.

Water holding capacity (WHC)

The filter paper press method recommended by Honikel (1987) was used to measure pressing loss (WHC). Meat samples, 2.5 cm in diameter and 1.0 cm in thickness, were collected and weighed. Each sample was placed on a humid filter paper between two Plexiglas plates and subjected to a certain weight pressure for 6 min. Then the weight press was removed, and the sample was reweighted. The WHC was determined by the difference between meat weights before and after pressing.

Microbiological examination

The microbiological examination of chicken meat samples from slaughtered birds, in terms of aerobic plate count (APC) and most probable numbers (MPN) of coliforms, fecal coliforms and *E. coli*, was done according to the methods recommended by the Association of Official Analytical Chemists (AOAC, 1990).

The profitability measures

Then comparative economic analysis between these trials according to the methods of Beg *et al*. (2011) and Ghosh *et al*. (2012) using the following equations:

Equation (4): Yield per unit area= sum of all birds' weight/unit

Equation (5): Production efficiency index (PEI) = [Body weight in Kg × (100 - % mortality)/Duration of fattening in days × feed conversion ratio] × 100.

Equation (6): Benefit-cost ratio (BCR) = (total revenue/m^2)/(total cost/m^2)

Equation (7): Net profit per unit area of floor space = (Income from selling birds – Total cost of production)/unit area of floor space.

Equation (8): Profitability index (PI) = Net Profit/Total Revenue

Total revenue: means the total income from selling the birds. While total costs including costs of purchasing the one-day-old chicks, pellet feed, sawdust as litter, energy costs, other material costs (drugs, vaccines and disinfectants) and labor costs.

Statistical analysis

Data were presented as mean ± standard error of means and analyzed by independent T-test and one-way ANOVA test using SPSS (SPSS statistical package, SPSS Inc., Chicago, IL, USA). Probability values less than 0.05 ($P<0.05$) were considered significant.

Results

Influence of stocking density and probiotic supplementation on bird performance

Regarding the effect of different stocking densities and probiotic supplementation of broiler chicken performance, CFI was not significantly affected by increasing density. Moreover, Protexin® probiotic supplementation significantly ($P<0.01$) increased the whole cycle FI in LSDP group while. It did not significantly affect the CFI in HSDP group (Table 1). Concerning the CBWG was significantly ($P<0.01$) decreased with increasing density, though, it is obvious that probiotic supplementation significantly ($P<0.01$) increased CBWG in LSDP and HSDP groups. There is a significant ($P<0.01$) poor CFCR with increasing density. Interestingly, probiotic supplementation improved CFCR in LSDP ($P<0.01$) and HSDP ($P<0.05$) groups (Table 1).

Table 1. The effect of different stocking densities and probiotic supplementation on cumulative feed intake (CFI), cumulative body weight gain (CBWG), and cumulative feed conversion ratio (CFCR) of broiler chickens.

Chicken groups	CFI	CBWG	CFCR
LSD	3771.31±12.77^b	2054.23±30.99^b	1.83±0.03^b
LSDP	3912.59±21.10^a	2247.68±37.50^a	1.74±0.02^c
HSD	3721.93±19.61^b	1837.00±19.94^c	2.03±0.01^a
HSDP	3791.07±7.02^b	1979.69±7.86^b	1.92±0.01^b

Results are expressed as means of at least 3 replicates ± standard error. Different small letter superscripts (a, b, c) within a column indicate significant differences between means. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/m^2) with probiotic supplementation.

Effect of stocking density and probiotic supplementation on blood stress indicators

Investigating the effect of stocking density and probiotic supplementation on blood stress indicators revealed that increasing stocking density elevated the serum corticosterone (CS) levels ($P<0.01$). On the other hand, however, probiotic supplementation had no significant effect on CS levels in LSDP group, it succeeded to decrease ($P<0.01$) CS levels in HSDP group (Table 2). As well as the levels of serum malondialdehyde showed that broilers reared at HSD did not pose oxidative stress as indicated by non-significant alteration in serum MDA levels. Moreover, probiotic supplementation did not significantly affect MDA levels at both densities (Table 2). Additionally, a reduced ($P<0.01$) mRNA expression of IL6 with increasing density was found. Whereas probiotic supplementation did not significantly affect mRNA expression at both densities (Table 2).

Impact of stocking density and probiotic supplementation on carcass and giblet yields

The obtained data revealed that increasing stocking density decreased (P<0.05) carcass yield (g) and dressing yield (P>0.05) but did not alter the total giblet weight. Also, the relative and absolute weights of spleen and bursa, as anatomical and immunological indicators of stress, were reduced by increasing the stocking density which indicates possible immunological stress on birds. On the other hand, probiotic supplementation had mostly positive effects on carcass weight, dressing yield, total giblet weight, and weights of spleen and bursa. That was particularly significant (P<0.05) in the case of absolute bursa weight in LSDP group. (Table 3).

Effect of stocking density and probiotic supplementation on carcass quality parameters

Concerning the carcass quality parameters in terms of muscle pH, DL, CL and WHC, we noticed that increasing the stocking density did not alter muscle pH. Nevertheless, high stocking density increased the water loss from broiler meat which was illustrated by the increase in the values of DL (P<0.01) and CL (P>0.05) and a decrease in WHC (P<0.05). Interestingly, the unfavorable effect of high stocking density on the water holding capacity of broiler muscles was reversed by probiotic supplementation. Given that probiotic supplementation decreased (P>0.05) DL, CL and increased WHC at LSDP group. Similar effects were noticeable at HSDP group for DL, WHC (P<0.05) as well as CL (P>0.05) (Table 4).

Regarding the effect of stocking density and probiotic supplementation on the sensory acceptability of broiler meat, remarkably, high stocking density diminished the overall acceptability of broiler meat which was clear from the reduction in the scores given by panelists to the color, texture (P>0.05), odor (P<0.05), taste and overall acceptability (P<0.01) of meat from groups reared under high stocking densities, however, the decrease in sensory attribute scores did not affect the meat grade (Table 4). Conversely, Protexin® probiotic supplementation did not badly affect the sensory acceptability of broiler meat, however, it increased the scores given by panelists to color, odor, texture and taste, which in total increased the overall acceptability at both normal and high stocking densities (Table 4).

Effect of stocking density and probiotic supplementation on carcass microbiological quality

In order to investigate the effect of the stocking density and probiotic supplementation on the microbiological quality of broiler meat, we determined the total bacterial counts (CFU/g) and MPN of coliforms, fecal coliforms and *E. coli*

Table 2. The effect of different stocking densities and probiotic supplementation on some stress indicators in blood of broiler chickens.

Chicken groups	Corticosterone levels (µg/dL)	Malondialdehyde levels (nmols/mL)	IL6 (mRNA expression)
LSD	0.99 ± 0.0^{c}	2.80 ± 0.09^{a}	1.00 ± 0.00^{a}
LSDP	0.98 ± 0.01^{c}	2.77 ± 0.33^{a}	1.52 ± 0.06^{a}
HSD	1.95 ± 0.01^{a}	3.99 ± 1.29^{a}	0.73 ± 0.07^{b}
HSDP	1.47 ± 0.01^{b}	3.93 ± 1.41^{a}	0.53 ± 0.03^{b}

Results are expressed as means of at least 3 replicates ± standard error.
Different small letter superscripts (a, b, c) within a column indicate significant differences between means. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/$m^{2)}$ with probiotic supplementation. IL6= Interleukin 6.

Table 3. Effect of different stocking densities and probiotic supplementation on carcass and giblet yields and some anatomical stress indicators of broiler chickens.

Chicken groups	Carcass weight (g)	Dressing yield (%)	Giblet weight		Spleen weight		Bursa weight	
			Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
LSD	1190.04 ± 16.37^{a}	69.48 ± 1.25^{a}	133.26 ± 2.17^{a}	7.79 ± 0.22^{a}	1.85 ± 0.14^{ab}	0.11 ± 0.01^{a}	1.05 ± 0.19^{b}	0.06 ± 0.01^{ab}
LSDP	1262.10 ± 20.68^{a}	72.15 ± 1.76^{a}	134.58 ± 3.77^{a}	7.71 ± 0.35^{a}	2.01 ± 0.21^{a}	0.12 ± 0.01^{a}	1.56 ± 0.12^{a}	0.09 ± 0.01^{a}
HSD	1093.08 ± 22.00^{b}	68.57 ± 2.05^{a}	127.31 ± 3.66^{a}	8.00 ± 0.35^{a}	1.37 ± 0.07^{b}	0.09 ± 0.01^{a}	0.73 ± 0.04^{b}	0.05 ± 0.01^{b}
HSDP	1178.10 ± 25.84^{ab}	69.35 ± 1.24^{a}	127.74 ± 2.86^{a}	7.54 ± 0.28^{a}	1.57 ± 0.08^{ab}	0.19 ± 0.01^{a}	0.92 ± 0.06^{b}	0.05 ± 0.01^{b}

Results are expressed as means of at least 3 replicates ± standard error.
Different small letter superscripts (a, b) within a column indicate significant differences between means. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/m^2) with probiotic supplementation.

Table 4. Effect of different stocking densities and probiotic supplementation on carcass quality parameters, sensory attributes and overall acceptability of broiler carcasses.

Chicken groups	pH	DL (%)	CL (%)	WHC (%)	Scores of sensory attributes and overall acceptability				
					Color	Texture	Odor	Taste	Overall acceptability
LSD	6.29 ± 0.09^{a}	6.01 ± 0.68^{c}	13.82 ± 3.48^{a}	35.33 ± 2.91^{a}	8.42 ± 0.08^{a}	8.33 ± 0.17^{a}	7.00 ± 0.00^{a}	8.17 ± 0.33^{a}	7.98 ± 0.15^{ab}
LSDP	6.08 ± 0.13^{a}	5.00 ± 0.47^{c}	9.00 ± 3.69^{a}	40.00 ± 1.15^{a}	8.50 ± 0.25^{a}	8.50 ± 0.01^{a}	8.00 ± 0.25^{a}	8.33 ± 0.09^{a}	8.33 ± 0.08^{a}
HSD	6.28 ± 0.21^{a}	13.48 ± 1.86^{a}	19.27 ± 0.46^{a}	24.67 ± 1.33^{b}	7.83 ± 0.17^{a}	8.00 ± 0.01^{a}	6.00 ± 0.00^{b}	6.60 ± 0.15^{b}	7.11 ± 0.05^{c}
HSDP	6.39 ± 0.29^{a}	7.05 ± 0.70^{bc}	14.57 ± 0.88^{a}	34.00 ± 1.15^{a}	8.17 ± 0.08^{a}	8.33 ± 0.17^{a}	6.50 ± 0.25^{b}	7.63 ± 0.09^{ab}	7.66 ± 0.13^{b}

Results are expressed as means of at least 3 replicates ± standard error.
Different small letter superscripts (a, b, c) within a column indicate significant differences between means. DL= Drip loss, CL= Cooking loss, and WHC= Water holding capacity. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/m^2) with probiotic supplementation.

(MPN/g) in meat samples from broilers grown at low and high stocking densities with or without probiotic supplementation. The obtained results showed that stocking density and probiotic supplementation did not induce significant effects in the total bacterial counts and coliforms MPN. On the contrary, probiotic supplementation significantly reduced ($p<0.05$) the MPN of both fecal coliforms and *E. coli* in broiler meat, particularly at high stocking density (HSDP group). While increasing stocking density was associated with significant elevations ($p<0.05$) in the MPN of both fecal coliforms and *E. coli* (Table 5).

Economic profitability of broiler production at different stocking densities with or without probiotic supplementation

Although increasing stocking density significantly increased the yield/unit area ($P<0.01$), BCR ($P<0.01$), net profit/unit area ($P<0.05$) and PI ($P<0.01$), there was a slight decrease in the PEI. This indicates a boost in economic efficiency with increasing stocking density. Whereas Protexin® supplementation at both stocking densities resulted in increased yield/unit area ($P<0.01$) and PEI ($P>0.05$), yet decreased BCR, net profit/ unit area, and PI (Table 6).

Discussion

Use of feed additives and water supplementation became a useful tool to maximize the productivity in poultry meat industry. The current study findings highlighted the efficacy of probiotics to overcome the impacts of high stocking density on broilers performance to improve productivity, carcass quality and profitability.

The observed results indicated that HSD impaired performance. HSD declined CBWG and CFCR. Similarly, Cengiz *et al*. (2015) found that increasing density from 10 to 20 bird/m^2 decreases the FCR between 0-42 days of age. However, Houshmand *et al*. (2012) and Tong *et al*. (2012) reported an improvement in the FCR during 1-42 days of age.

The reported poor performance induced by HSD may be due to the decline of voluntary FI in the last 3 weeks of the growing cycle with increasing age and stocking density (Zulkifli *et al*., 2009). HSD restrict the movement of birds that they could not access the feed and water (Cengiz *et al*., 2015). In addition, HSD leads to heat stress, which decreases the FI (Bessei, 2006). Moreover, the crowding induces nervousness resulted in reduction of feed consumption (Olowe, 2001). Furthermore, litter quality was deteriorated, which increased bacterial fermentation, moisture content (Yadgari *et al*., 2006) and ammonia volatilization leading to suppress birds' growth (Yadgari *et al*., 2006) by the significant damage to intestinal mucosal cells (Yeo and Kim, 1997) and interfered intestinal microbiota of broilers (Cressman *et al*., 2010). All the above-mentioned effects of HSD led to impairment of bird's health and suppress growth performance.

Data from this study revealed that protexin® probiotic supplementation enhanced performance in LSDP expressed by increasing FI. Likewise, Habibi *et al*. (2013) found that Protexin® probiotic improved FI from 0-42 days of age. On the contrary, Cengiz *et al*. (2015) and Souzaa *et al*. (2018) reported that the whole cycle FI was not significantly affected by probiotic supplementation. Furthermore, probiotic supplementation increased the CBWG in LSDP and HSDP groups. These results were supported by Cesare *et al*. (2017) and Habibi *et al*. (2013), who reported that Protexin® probiotic supplementation enhanced CFCR. In addition, Protexin® supplementation found to mitigate adverse effect of HSD on performance (Ramos *et al*., 2014). On the contrary, Cengiz *et al*. (2015) stated that the use of probiotics at HSD did not significantly affect WG.

The observed improvement in performance may be due to the efficacy of probiotic to secrete different enzymes that aid in the digestion of nutrients (J´ozefiak *et al*., 2004), increase digestive enzyme activity, which enhance digestibility of protein and starch, (Wang and Gu, 2010), increase the villi height (Bai *et al*., 2013), enhance the absorption of nutrient (Caspary, 1992) improve intestinal health (Flores *et al*., 2016) and develop intestinal mucosa of broilers (Fallah *et al*., 2013) as well as stimulating appetite (Nahashon *et al*., 1992). All these factors contribute to increased food consumption and digestibility of the diet (Shim *et al*., 2010), which improve the productive performance.

Table 5. Effect of rearing densities and probiotic supplementation on microbiological status (CFU or MPN/ g muscle) of broilers` carcasses.

Chicken groups	Aerobic plate count (CFU/g)	Coliforms (MPN/g)	Faecal coliforms (MPN/g)	*E. coli* (MPN/g)
LSD	$1.75\times10^5 \pm 1.8\times10^{4a}$	$1.10\times10^4 \pm 0.00^a$	$6.20\times10 \pm 4.52\times10^b$	$2.46\times10 \pm 1.44\times10^b$
LSDP	$1.66\times10^5 \pm 1.7\times10^{4a}$	$1.10\times10^4 \pm 0.00^a$	$1.67\times10^2 \pm 3.77\times10^{ab}$	$2.40\times10 \pm 1.20\times10^b$
HSD	$1.82\times10^5 \pm 1.1\times10^{4a}$	$1.10\times10^4 \pm 0.00^a$	$2.10\times10^2 \pm 3.78\times10^a$	$1.03\times10^2 \pm 4.95\times10^a$
HSDP	$1.86\times10^5 \pm 1.5\times10^{4a}$	$1.10\times10^4 \pm 0.00^a$	$9.10\times10 \pm 5.83\times10^b$	$3.66\times10 \pm 3.66\times10^b$

Results are expressed as means of at least 3 replicates ± standard error.
Different small letter superscripts (a, b) within a column indicate significant differences between means. CFU= colony forming unit, MPN- most probable number. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/m^2) with probiotic supplementation.

Table 6. Economic efficiency of rearing broilers under LSD and HSD with or without probiotic supplementation.

Chicken groups	Yield (Kg/unit area)	PEI	BCR	Net profit/ unit area /bird (LE/bird)	PI/ unit area /bird (LE/bird)
LSD	35.14 ± 0.48^d	216.70 ± 17.00^{ab}	1.48 ± 0.03^b	6.67 ± 0.31^b	0.20 ± 0.01^b
LSDP	38.23 ± 0.60^c	271.13 ± 25.08^a	1.11 ± 0.02^d	2.17 ± 0.32^d	0.06 ± 0.01^d
HSD	47.47 ± 0.44^b	154.13 ± 8.89^b	1.78 ± 0.01^a	8.05 ± 0.14^a	0.27 ± 0.00^a
HSDP	50.90 ± 0.21^a	182.99 ± 7.20^b	1.28 ± 0.00^c	4.41 ± 0.08^c	0.14 ± 0.00^c

Results are expressed as means of at least 3 replicates ± standard error.
Different small letter superscripts (a, b, c, d) within a column indicate significant differences between means. PEI= production efficiency index, BCR=benefit cost ratio, PI= profitability index. LSD = low density (10 birds/m^2) without probiotic supplementation (control). LSDP = low density (10 birds/m^2) with probiotic supplementation. HSD = high density (15 birds/m^2) without probiotic supplementation. HSDP = high density (15 birds/m^2) with probiotic supplementation.

The increased blood CS caused by HSD indicated the stressful effect of overcrowdings on broilers. This data was in harmony with Najafi *et al*. (2015) and disagreeable with Houshmand *et al*. (2012) and Cengiz *et al*. (2015), who stated that HSD did not affect blood CS. The increased CS levels may be attributed to the competition on feed and water (Craig *et al*., 1986), the heat stress (Rashidi *et al*., 2010) and increasing litter moisture and ammonia (Dawkins *et al*., 2004) caused by rearing at HSD.

The reduced mRNA expression of IL6 with increasing density suggested that the stress induced by HSD could impair bird immunity. This finding is in harmony with that reported by Munck *et al*. (1984) while, it unlike that found by Kang *et al*. (2011). The obtained result confirmed that the decrease in space allowance is a stressful condition that can negatively affect the animal's immune system and ability to overcome viral and bacterial infections (Feddes *et al*., 2002; Estevez, 2007). Stressors can increase the release of the stress hormone, namely CS, which has inhibitory effects on some immune functions including lymphocyte proliferation, the production of cytokines and immunoglobulins, anti-inflammatory agents, and cytotoxicity (Munck *et al*., 1984).

The reported decrease in carcass yield as a result of HSD in this study was also announced by Simitzis *et al*. (2012), who declared that increasing stocking density significantly decreased eviscerated carcass weight. On the other hand, carcass weight was not significantly affected by increasing density (Abouelenien *et al*., 2016). Moreover, the results of dressing % in this study were similar to the findings of Gabanakgosi *et al*. (2014) and Cengiz *et al*. (2015), who recorded that upon processing, stocking density did not influence carcass yield relative to BW. However, Farhadi *et al*. (2016) reported an increase in the dressing yield HSD. Furthermore, the prominent increase in the total giblet weight by increasing density was in harmony with Turkyilmaz (2008) and Jayalakshmi *et al*. (2009). The observed non-significant drop in the weights of lymphoid organs was to some extent in agreement with Farhadi *et al*. (2016) noticed a non-significant reduction in the spleen weight. Moreover, they were in harmony with the findings of Cengiz *et al*. (2015), who found an insignificant effect of HSD on lymphoid organs` weights.

The improvement in carcass and giblet yields induced by Protexin® supplementation came in parallel with Shabani *et al*. (2012) and Habibi *et al*. (2013), who observed significantly higher carcass yields with probiotic supplementation. Moreover, Protexin® supplementation significantly improved the absolute bursa weight at LSD group, which agrees with Willis *et al*. (2007), who reported an improved bursa weight with probiotic supplementation, which is considered an improvement in the immune system (Nourmohammadi *et al*., 2011).

The absence of stocking density effect on meat pH in this study was in accordance with the findings of Tong *et al*. (2012) and Simitzis *et al*. (2012), who declared no change in the meat pH with increasing density. Conversely, the negative effects of high stocking density on carcass quality, which was evident by the rise in drip loss and cooking loss with a decrease in WHC of broiler meat, were not in the case with Moreira *et al*. (2004) and Simitzis *et al*. (2012), who reported no significant change in the cooking loss with increasing density. On the other hand, the decreased DL, CL and increased WHC at both densities in the case of Protexin® supplementation were also reported by Zhou *et al*. (2010) and Park and Kim (2014), who found an improvement in the DL and WHC when probiotics were used. In context, Popova (2017) assumed that the feeding regimes with probiotics exhibit as a natural prospective to improve poultry meat quality in vivo due to the enhancement in the intestinal microbiota and the reduction of pathogenic bacteria intestinal load, which in turn improve the birds' health and performance as well as meat quality.

Concerning the sensory attributes, the indifference in the overall acceptability with increasing density was in harmony with reports of Thomas *et al*. (2004) who found that overall acceptability was not affected by density. In contrast, Protexin® supplementation improved the overall acceptability score at both densities reported by Mahajan *et al*. (2000), who revealed that overall organoleptic scores in terms of appearance, texture, juiciness and overall acceptability were higher in probiotic fed broilers than counterparts fed with traditional basal diet. The meat color is crucial for the consumer assessment of meat freshness and quality and is determined by measuring the lightness (L*), redness (a*) and yellowness (b*) of meat (Kadim and Mahgoub, 2013). Similar to results from the present study, the use of probiotics in the water of broilers significantly increased the redness in the breast, while there was no effect on the yellowness and lightness in breast and thighs of birds (Haščík *et al*., 2015), since the redness is most favored by consumers and lower yellowness values indicate less pale meat (Jiang *et al*., 2014).

As has already been outlined, it is clear that increasing stocking density decreased carcass quality in term of decreased carcass yield, overall acceptability, water loss from broiler meat which adversely may affect meat tenderness and flavor. The decreased carcass quality by increasing density may have resulted from the poor micro-environmental conditions inside the poultry house, competition for feed and water, increased litter moisture condition, elevated ammonia level due to degradation of uric acid by the microorganisms and other various pollutants (Jayalakshmi *et al*., 2009; Souzaa *et al*., 2018). The reduction in feed intake and decrease in BWG may be linked to decreased carcass yield (Dozier *et al*., 2005). On the contrary, the improved carcass quality with probiotic supplementation may be linked to the growth-promoting effect of probiotics (Jin *et al*., 1998) that may have contributed to the relatively improved carcass yield. Additionally, the claim of increased fat digestibility with probiotic supplementation resulting in high-fat content of the meat that widely contributed to taste, odor, flavor, tenderness (Endo and Nakano,1999) may be the reason of improving overall acceptability score and improved carcass quality.

In parallel with the obtained findings that probiotic supplementation improved the microbiological quality of broiler carcasses, Lavipan probiotic product contributed to the reduction of Campylobacter spp. count in poultry carcass (Smialek *et al*., 2018). They attributed this result to the fact that probiotics reduce the degree of intestine invasion with bacteria since carcass contamination with bacteria is directly related to the degree of bacteria migration from the intestine to muscles (Hue *et al*., 2011). So, we postulate that the reduction in the MPN of fecal coliforms and *E. coli* in broiler carcasses received probiotic supplementation could be ascribed to the reduction of bacteria migration from the intestine to muscles by the effect of probiotics.

The hypothesis that the economic benefit per square meter was often still higher if the chickens were stocked more densely (Cravener *et al*., 1992; Feddes *et al*., 2002) was confirmed in our study. However, Ghosh *et al*. (2012) found poor production efficiency index, profitability index and total revenue with increasing density. In addition, Lallo *et al*. (2012) recorded that 10 birds/m^2 was the optimal stocking density giving the best economic returns. The increased economic efficiency at HSD may be owing to the production of more kilograms per unit area and the reduced fixed cost of production (Puron *et al*., 1995).

In the current study, Protexin® supplementation impaired the economic returns by decreasing BCR, net profit per unit area and PI. These data were in consistent with that obtained

by da Silva *et al*. (2011) and Nunes *et al*. (2012). On contrary, probiotic supplementation improved the economic efficiency and profitability (Hooge *et al*., 2003; Timmerman *et al*., 2006; Habibi *et al*., 2013).

The observed enhancement in the BW by Protexin® supplementation was not enough to face and neutralize the cost of the Protexin®. in addition, the increased FI with Protexin® supplementation and the subsequent increase in the feed cost may be the causal factors of decreased economic efficiency.

Conclusion

HSD posed a stressful effect on birds expressed by increasing blood CS, decreasing mRNA expression of IL6 and reduced carcass weight, dressing yield, giblet yield, carcass quality parameters and sensory acceptability. On the other hand, Protexin® probiotic supplementation reversed the effect of HSD on blood CS, improved performance, carcass quality and sensory acceptability of broilers. Additionally, probiotics inhibited the growth of pathogenic bacteria, such as *E. coli*, in the gastrointestinal tract of broilers through competence exclusion. Thus, probiotics could be exploited as an alternative method for the reduction of poultry product contamination with bacteria.

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Conflict of interest

Authors declared that there is no conflict of interests exist.

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