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Influence of *Moringa Oleifera* leaf extract and synbiotic supplementation on mitigating stress of broilers subjected to high stocking density

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ABSTRACT

Environmental factors such as stocking density can cause stress and negatively affect the physiological status and meat quality of broiler chickens. In the current study, we evaluated the effects of increased rearing density on the growth performance, stress, fear levels, meat quality and liver histopathology of broilers as well as suggesting a use of different supplementations that may be helpful to reverse such adverse effects. Three hundred sixty one day-old cobb broilers (44.0±5.24 g live-weights) were randomly distributed to 24 replicate pens to create two different stocking densities (D10 and D15 bird/m^2) with the different supplementation treatments: 1) 10 birds/m^2/pen (without any treatment, control, "CD10"), 2) 10 birds/m^2/pen (Moringa aqueous extract treatment "MAED10"), 3) 10 birds/m^2/pen (synbiotic "SynD10"), 4)15 birds/m^2/pen (without any treatment control SD15 "CD15"), 5) 15 birds/m^2/pen (Moringa aqueous extract treatment "MAED15"), 6) 15 birds/m^2/pen (synbiotic "SynD15"). Results of the current study revealed that there was a significant increase in the overall experimental period body weight gain ($P<0.01$) with increasing density without affecting feed intake, body weight and feed conversion ratio. High stocking density resulted in significantly increased corticosterone ($P>0.001$), altered brain monoamines, deteriorated some carcass quality parameters and declined liver health. Additionally, it was concluded that Moringa aqueous extract (MAE) and synbiotic (Syn) supplementation have a significant effect in reversing the catastrophic effect of high stocking density (SD) with minimal effect at the lower density groups.

Introduction

Poultry production remains the widest spread of all livestock enterprises (Hussein and Jassim, 2019) that plays an important role in meeting the nutritional needs of the community (Astuti and Suripta, 2020). The rapid production and growth capability of broilers make them vulnerable to stressful environments, currently consumers demand for high quality products, so they are aware of animal welfare and quality (Astuti and Suripta, 2020; Nasr *et al*., 2021).

The increased demand for poultry meat force producers to follow various strategies for amplifying the production such as increasing the production per unit area (increasing stocking density), using dietary inclusions as antibiotics and other alternatives. Intensive rearing (high stocking density) is a management routine intended for (practiced to) decreasing cost related with labor, fuel, housing, and equipment, enhancing profitability due to higher chicken production per fixed stocking area (Goo *et al*., 2019) but it is assumed as a factor of stress (Nasr *et al*., 2021) that may have detrimental effect on poultry health, immune system, welfare, and productive performance (Houshmand *et al*., 2012) especially during the finisher phase when the body weight per unit area ratio is very high (Thema *et al*., 2022). The crucial aim of global poultry industry is not only to amplify the production of broilers meat (kg) per m^2 with superior uniformity and quality but also to prevent production losses caused by overcapacity and the possible decrease in the product quality (Nasr *et al*., 2021).

Antibiotics have been used at subtherapeutic concentrations " Subtherapeutic antibiotics" as feed additives reduce inflammation (Shin *et al*., 2020), prevent infectious diseases and to promote growth performance by increase the rate of weight gain and/or the efficiency of feed utilization in animals (Patel *et al*., 2020). Even so, their use created a public concern about bacterial resistance and residues in meat products (Kirchhelle *et al*., 2020; Nadeem *et al*., 2020). Eliminating antibiotics in poultry production with no reliable alternatives has raised noticeable consequence including comprised productive performance and increased infectious diseases incidence and associated mortality (Cervantes, 2015). Several alternatives of non-therapeutic antibiotics have been tested, where prebiotics and probiotics are suitable (Redweik *et al*., 2020). A synbiotic is a mixture of probiotics and prebiotics that aids the host by improving the survival and activity of beneficial microorganisms in the gut" (Gyawali *et al*., 2019). The beneficial effects of supplementing synbiotic include improvement of growth performance, gut integrity, and immune function to increase production and health in broilers with and without stress challenges (Yan *et al*., 2019; Bogucka *et al*., 2019; Rostagno, 2020).

Many herbal plants have been used to replace antibiotics in poultry production (Hafeez *et al*., 2020). *Moringa Oleifera* is an excellent source of nutritional, therapeutic and industrial tool for human and livestock ailments (Mahmoud *et al*., 2019), it contains alkaloids, flavonoids, anthraquinones, vitamins, glycosides, and terpenes. In addition, novel isolates such as muramoside A&B and niazimin A&B have been identified in the plant and have potent antioxidant, anticancer, antihypertensive, hepatoprotective, and nutritional effects (Pareek *et al*., 2023).

Based to our knowledge, few studies have examined and compared the effects of both synbiotic (SYN) and Moringa aqueous extract (MAE) on performance and welfare of broilers reared under two different densities in uncontrolled environment. We hypothesized that MAE and SYN supplementation may decrease the possible adverse effects of increasing density, so the objective of the current study is to evaluate and compare the effect of both MAE and SYN on broiler performance meat quality and

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various welfare indicators.

Materials and methods

This study was conducted in the poultry house of Animal and Poultry Management and Wealth Development Department at the Faculty of Veterinary Medicine Beni-Suef University, Egypt to investigate the effect of Moringa aqueous extract and synbiotic supplementation on welfare of broilers reared under two different stocking densities (SD).

Chemicals, Moringa aqueous extract and Synbiotic

Sento-bact synbiotic®, (Lactic acid bacteria culture; Lactobacillus Acidophilus 50% 1.0×10^8 CFU/g, lactobacillus planterum 49% 9.8×10^7 CFU/g, Bifidobacterium Bifidum 1% 2.0×10^6 CFU; Enzyme blend concentrate; Amylase 25 units/g, Cellulose 4.50 units/g Beta-gluconase 2.25 units/g, Hemicellulase 2.75 units/g; Bacillus Subtlis fermentation extract (L- lactate) 1.00g/kg; Aspergillus Niger fermentation extract (source of protease) 1.00g/kg; Vitamin C 10.00g/kg; Dextrose up to 1 kg) purchased from Pharma Swede pharmaceutical Company, Cairo, Egypt.

Moringa Oleifera leaves aqueous extract, dried *Moringa Oleifera* leaves were ground into fine particles and soaked in water for 24 hours then filtrated using muslin cloth piece to separate the debris, this process was done daily. The extract was added to water and introduced to birds at rate of 90 ml/L according to Alabi *et al*. (2020) and Egbu *et al*. (2022).

Corticosterone (CS) and chemicals for measuring monoamines were obtained from Sigma Aldrich.

Birds' accommodation, grouping and supplementation

A total number of 360 unsexed one day old (Cobb type breed) chicks, purchased from a commercial hatchery at Beni-Suef governorate. The chicks were brooded at 33ºC using electric heaters for the first week of age, then were randomly distributed at the end of the first week into six groups with four replicates of each in 24 floor pens - each measured 1×1.6 m- with a new wood shaving litter material to overcome the possible deteriorated air and litter quality that occur during the brooding period as in Table 1.

All birds were reared under the same environmental conditions; the ventilation was maintained using windows, fans, and exhaust fans. Heating was performed by the electric heaters, with a decrease in the temperature 2ºC each week. Lighting program was set as continuous lighting for the first week and 23-hour light and 1 hour dark till the end of the experiment by regularly distributed red bulbs.

Feed and water were provided Ad libitum using manual plastic feeders and drinkers. The birds were fed using a two-phase broiler-feeding regime. This consisted of a starter containing 23% protein crumble for the first 21 days, followed by a grower pellet with 21% protein until the end of the study at day 42. Study was approved by the institutional animal care and use committee (ICAUC), Beni-Suef University and assigned number 022-505.

Sampling

Blood sample, five ml of blood sample was collected from the wing vein of five randomly chosen birds per replicate (20 birds per treatment), that were fastened overnight, without anticoagulant for serum separation, the samples were collected weekly for corticosterone. Samples were centrifuged at 3000-4000 r.p.m for 10-15 minutes, serum was aspirated and put in clean Eppendorf tubes and frozen in deep freezer at -20°C until analysis.

Organ samples, at the 6th wk. of the growing cycle five randomly chosen birds per replicate were fasted overnight and sacrificed, their brains were removed quickly and placed in iced normal saline, perfused with the same solution to remove blood cells, plotted on filter paper and frozen at -80°C until used for estimation of the biochemical parameters. Additionally, liver samples were taken for histopathological investigation.

Measurements

Performance

Feed intake (FI)

The diets were offered daily at fixed time in the morning, then the feed intake was calculated according to Beg *et al*. (2011). Broilers weight (wt.) and weight gain (WG) were recorded weekly.
Feed conversion ratio (FCR) was recorded weekly, FCR= FI (g")/BWG (g)

Physiological indicators of stress

Serum corticosterone level was measured using commercial ELISA kits (Sigma Aldrich)

Determination of brain monoamines was done according to method by Abdel-Salam et al. (2011)

Fear response "fearfulness", birds were tested for tonic immobility, a well validated fear test (Forkman *et al*, 2007), five birds/ replicate were caught and carried in an upright position to a separate neighboring room. A few seconds after the bird was caught, tonic immobility was induced by placing the bird on its back with the head hanging in a U-shaped wooden cradle (Jones and Faure, 1981). The bird was restrained for 10 s. The observer sat in full view of the bird, about 1 m away, and fixed his eyes on the bird to give the fear-inducing properties of eye contact. If the bird remained immobile for 10 s after the experimenter removed his hands, a stopwatch was started to record latency Duration (s) until the bird righted itself. If the bird righted itself in less than 10 s, then it was considered that tonic immobility had not been induced, and the restraint procedure was repeated (3 times maximum). If the bird did not show a righting response over the 10-min test period, a maximum score of 600 s was given for righting time. Thus, tonic immobility duration ranged from 0 to 600 s.

Table 1. Illustrate bird grouping, treatments and rearing densities.

Bird grouping	Rearing density	Number of birds
Group1: Control group (CD10),		
Group 2: *Moringa Oleifera* leaves aqueous extract group (MAED10)	10 birds/m^2	48 birds (12 bird /replicate)
Group 3: Sentobact® treated birds (SynD10)		
Group 4: Control group (CD15),		
Group 5: *Moringa Oleifera* leaves aqueous extract group (MAED15)	15 bird/m^2	72 birds (18 bird/ replicate)
Group 6: Sentobact® treated birds (SynD15)		

Carcass quality measures

Dressing percentage

At the 6th week of the growing cycle five birds/ replicate were slaughtered for determination of dressing percent according to Beg *et al.* (2011).

Drip loss (DL)

The drip loss was determined according to the method recommended by Kauffman *et al.* (1986). Recording the pH of the meat, the time postmortem and the age of the birds is very important when evaluating the results of drip loss.

A slice of muscle (skinless Pectoralis major muscle) of about 2.5 cm thickness and 50-100 g weight was removed from the chicken breast. Room temperature during cutting was similar to the temperature of the meat. The slice of muscle was weighted and suspended by means of 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 measuring period the muscle was taken from the pouch, dried gently with an absorbing tissue and reweighed. During weighing, care was taken that no condensation of water vapour occurs on the cold surface. Drip loss was expressed as the weight loss (as the percentage of original weight).

Cooking loss (CL)

Following the drip loss determination, the same sample was used immediately for cooking loss measurements. If there was a delay before cooking loss measurement, the sample was 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 the portable needle-tipped 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). Samples, 2.5 cm in diameter and 1.0 cm in thickness, were collected and weighed. A sample was placed on humid filter paper between tow Plexiglas plates and subjected to a specified pressure for 6 min. The smaller inner ring is the pressed meat ring, and the larger outer circle is the boundary of fluid expressed from the meat at a given force (Figure X). Then the weight was removed, and the sample was reweighted. The WHC was determined by the difference between the meat weight before and after pressing. This method requires some knowledge of the history of the sample (post-mortem time, pH) for evaluation of the results. The filter paper press method is not highly correlated to drip loss but has been a useful relative indication of WHC.

Histopathological examination

All collected samples were examined histopathologically using formalin-fixed paraffin-embedded (FFPE) technique (Bancroft and Gamble, 2008). Briefly, samples were fixed in a 10% formalin solution for at least 24 hours. All sections were dehydrated in ascending grades of alcohol (ethanol) 70, 80, 95% and absolute alcohol. The tissues were then cleared in xylene and embedded in paraffin wax. Serial sections of (5 µm) thickness were cut using a rotary microtome and mounted on glass slides. The sections were deparaffinized, hydrated and stained using the routine hematoxylin and eosin staining (H&E) method (Bancroft and Gamble, 2008). After histopathological examination using Leica light microscopy, photomicrographs of selected slides from all experimental groups were taken under different magnifications using an automated built-in digital camera (Leica, Germany).

Statistical analysis

Data were presented as mean ± standard error of means and analyzed by independent T-test and one-way ANOVA test using SPSS (Statistical Package for Social Science. 2011). Probability values less than 0.05 ($P<0.05$) were considered significant.

Results

Regarding the Effect of Moringa aqueous extract and synbiotic supplementation on broiler performance at two different rearing densities

Broiler performance was adversely affected by increasing rearing density from 10 to 15 bird/m^2 especially the weight gain ($P<0.01$) as shown in Table 2. On the other hand, it was noticed that Moringa and synbiotic supplementation didn't improve broiler performance at both densities.

Table 2. The effect of Moringa aqueous extract and synbiotic supplementation on broiler performance at two different rearing densities.

Groups \ Performance		FI (g)	WT (g)	WG (g)	FCR
CD10		862.57±121.74	1529.98±341.36	520.27±38.07	1.74±0.29
MAECD10		882.43±122.84	1597.31±382.61	569.89±34.32	1.54±0.15
SynD10		865.37±117.89	1503.13±331.41	509.96±12.51	1.71±0.23
CD15		795.03±87.61	1308.91±241.04	397.55±56.81	2.25±0.51
MAECD15		836.97±97.05	1438.21±303.82	469.64±23.85	1.86±0.28
SynD15		835.23±99.15	1418.87±303.37	468.90±4.578	1.84±0.24
	SD	NS	NS	**	NS
Significance	D10.ttt	NS	NS	NS	NS
	D15.ttt	NS	NS	NS	NS

Results were expressed as means ± standard error.
*Indicate significance within columns ($P<0.01$)
FI: Feed intake, WT:weight, WG: Weight gain, FCR: Feed conversion ratio, CD10: Control group density 10 birds/m^2, MAED10: Moringa aqueous extract density 10 birds/m^2, SynbD10: synbiotic density 10 birds/m^2, CD15: control group density 15 birds/m^2, MAED15: Moringa aqueous extract density 15 birds/m^2, SynbD15: Synbiotic density 15 birds/m^2, SD: Stocking density, D10.ttt: 10 birds/m^2 treatment, D15.ttt: 15 birds/m^2 treatment, NS: Nonsignificant

The effect of rearing density, moringa aqueous extract and synbiotics supplementation on some quality parameters of broiler carcasses

Concerning the dressing percent, it was clear that increasing SD adversely affect dressing percentage (P>0.001) (Table 3). Conversely, neither Moringa nor synbiotic supplementation alter dressing percentage at both densities despite the non-significant improvement at high density.

Regarding the carcass quality parameters in terms of muscle pH, DL, CL and WHC, we noticed that meat pH wasn't affected by stocking density or treatments. It was remarked that high stocking density increased the water loss from broiler meat which was illustrated by the increase in the values of DL (p<0.001) and CL (p>0.001) and a decrease in WHC (p<0.05). Additionally, synbiotic supplementation achieved a significant (p<0.001) decrease in CL at normal density as compared with MA supplementation. Interestingly, the unfavorable effect of high stocking density on the water holding capacity of broiler muscles was reversed by both synbiotic and MA supplementation as the DL and CL were significantly (p<0.001) decreased by both treatments. (Table 3).

Influence of stocking density, moringa aqueous extract and synbiotics supplementation on stress indicators

The forementioned results in Table 4 clarified that increasing SD significantly (P>0.001) increased serum corticosterone levels. On the contrary, MA significantly (P>0.001) decreased corticosterone levels at HD as compared with CHD group without any significance with SHD group.

It was evident that increasing SD significantly (P>0.001) increased NE & DA and SE levels. Both MA and synbiotic supplementation significantly (P<0.001) improved the altered NE, DA and SE levels at HD groups only.

Effect of Moringa aqueous extract and synbiotics supplementation on fear response

Investigating the effect of Moringa aqueous extract and synbiotic supplementation on broiler behaviour revealed that increasing stocking density increased fearfulness as indicated by significant (P>0.001) increase in tonic immobility (TI) duration. on the other hand, Moringa aqueous and synbiotic supplementation significantly (P>0.001) decreased TI duration and decrease fearfulness at HSD only (Fig. 1).

Influence of stocking density, moringa aqueous extract and synbiotic supplementation on liver pathology

Most livers from all experimental groups revealed normal hepatic architecture. Briefly, normal central veins are present and normal hepatocytes are arranged in cords (Figure 2.a). The fatty change also was detected in the liver of some birds, increasing SD increased Fatty change, the total histopathological score (THPS) (p>0.05) and Bile duct hyperplasia (p<0.05) Table 5. Chronic cholangiohepatitis was observed in overstocked broilers as well (Figure 2.b) that characterized by extensive proliferation of bile ductules with fibrosis. Hepatocytes were compressed and suffered a necrotic change. Multiple granulomas were observed among the affected liver (Figure 2.c), characterized by proliferation and infiltration of lymph-plasmacytic infiltrate. A fibrous connective tissue capsule sepa-

Table 3. Effect of rearing density, Moringa aqueous extract and synbiotic supplementation on some quality parameters of broiler carcasses:

Groups / Carcass quality	Dressing (%)	pH	DL	CL	WHC
CD10	77.69 ±3.26	5.74 ± 0.04	4.62±0.72	29.89±0.81	24.52±2.00
MAECD10	81.98 ±2.75	5.54±0.16	4.85±0.37	33.19±1.08	27.08±3.01
SynD10	75.42 ±3.77	5.65±0.06	3.88±0.65	26.66±2.04	24.22±0.62
CD15	67.39 ±1.11	5.76±0.08	16.61±2.79	52.79±3.35	15.33±2.54
MAECD15	71.9 ±0.94	5.68±0.08	8.37±0.70	36.35±2.06	16.28±1.03
SynD15	73.73 ±1.24	5.59±0.09	8.21±0.82	31.09±2.72	18.49±1.90
SD	***	NS	***	***	*
Significance D10.ttt	NS	NS	NS	***	NS
D15.ttt	NS	NS	***	**	NS

Results were expressed as means ± standard error.
*,**,***indicate significance within columns (P<0.05, P<0.01, P<0.001respectively). DL: Drip loss, CL: Cooking loss, WHC: Water holding capacity, CD10: Control group density 10 birds/m^2, MAED10: Moringa aqueous extract density 10 birds/m^2, SynbD10: synbiotic density 10 birds/m^2, CD15: control group density 15 birds/m^2, MAED15: Moringa aqueous extract density 15 birds/m^2, SynbD15: Synbiotic density 15 birds/m^2, SD: Stocking density, D10.ttt: 10 birds/m^2 treatment, D15.ttt: 15 birds/m^2 treatment, NS: Nonsignificant.

Table 4. Effect of Moringa aqueous extract and synbiotic supplementation on some stress indicators of broiler chickens reared at two different densities.

	Corticosterone (µg/dL)	Monoamines (nmols/ml)		
		NE	DA	SE
CD10	0.47 ± 0.03	186.90±6.52	450.50±54.56	72.57±2.25
MAECD10	0.51±0.04	165.85±19.02	408.17±66.44	76.63±5.97
SynD10	0.72±0.10	167.83±16.54	510.67±42.28	80.56±5.48
CD15	1.78±0.19	355.16±29.94	904.33±2.85	125.47±5.16
MAECD15	1.04±0.26	282.23±14.85	612.02±30.23	102.63±10.88
SynD15	1.38±0.23	296.18±16.70	661.23±71.65	99.33±3.83
SD	***	***	***	***
Significance D10.ttt	NS	NS	NS	NS
D15.ttt	***	***	***	***

Results were expressed as means ± standard error.
*: indicate significance within columns (P<0.001). NE: Norepinephrine, DA: Dopamine, SE: Serotonin, CD10: Control group density 10 birds/m^2, MAED10: Moringa aqueous extract density 10 birds/m^2, SynbD10: synbiotic density 10 birds/m^2, CD15: control group density 15 birds/m^2, MAED15: Moringa aqueous extract density 15 birds/m^2, SynbD15: Synbiotic density 15 birds/m^2, SD: Stocking density, D10.ttt: 10 birds/m^2 treatment, D15.ttt: 15 birds/m^2 treatment, NS: Nonsignificant.

rates the granulomatous reaction from the neighbouring hepatic parenchyma. The adjacent hepatocytes suffer from necrotic changes.

Moringa aqueous extract improved THPS at both densities ($p<0.05$ and $p<0.01$ at ND and HD respectively) and decreased fatty changes in HD group ($p<0.01$). while synbiotic decreased ($p<0.01$) THPS at HD group only.

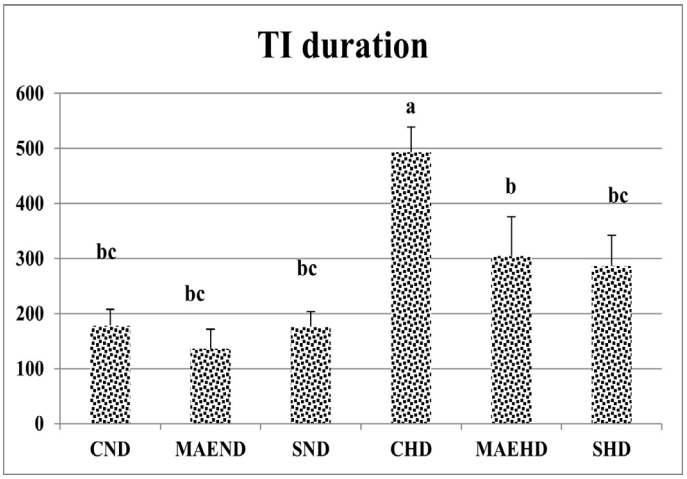

Fig. 1. The effect of Moringa aqueous extract and synbiotic supplementation on tonic immobility duration of broilers reared at two different densities.
Different superscript letters (a,b,c) indicate significance. CD10: Control group density 10 birds/m^2, MAED10: Moringa aqueous extract density 10 birds/m^2, SynbD10: synbiotic density 10 birds/m^2, CD15: control group density 15 birds/m^2, MAED15: Moringa aqueous extract density 15 birds/m^2, SynbD15: Synbiotic density 15 birds/m^2, SD: Stocking density, D10.ttt: 10 birds/m^2 treatment, D15.ttt: 15 birds/m^2 treatment.

Discussion

The current study investigated the effect of *Moringa Oleifera* aqueous extract and synbiotic supplementation on growth performance, carcass quality, fear and stress response and liver pathological changes of broilers reared at two different stocking densities.

Conflicting results about the effect of stocking density on the growth performance of broilers. Several authors have detected a significant reduction of growth performance with increasing stocking density (Chegini *et al*., 2018; Nasr *et al*., 2021; Thema *et al*., 2022). While others have stated that stocking density did not affect growth performance (Obeidat *et al*., 2019). Our results agreed with the majority of studies that mentioned increasing stocking density had a detrimental impact on growth performance represented in significant reduction in the average weekly weight gain which was also reported by Son *et al*. (2022).

The reduced performance with increasing density may be attributed to increased stress (Nasr *et al*., 2021) which accelerates corticosterone release that cause a restriction of glucose utilization, therefore growth in the form of protein accretion is reduced (Carsia, 2015). Stress enhances the metabolic rate causing an unfavorable effect on growth performances (Deeb *et al*., 2002), it redirects blood flows from the gastrointestinal tract to the peripheral tissues resulted in reduction of the nutrient and oxygen supply that harms gut and intestine health, performance (Lambert, 2009) and compromised nutrient absorption of broilers (Shakeri *et al*., 2014). Moreover, the reduction of body weight gain may be due to interfering with breast muscle hypertrophy and differentiation through regulating the expression of IGF-I, MyoD, and MSTN (Li *et al*., 2019).

Mixed findings about the effect of *Moringa Oleifera* aqueous extract (MOAE) on performance in broiler have been reported previously. Inclusion of that *M. Oleifera* aqueous extract proved to improve the performance (total feed intake, BW, BWG and FCR) of broilers (Hussein and Jassim 2019) and be used to replace antibiotic growth promoter (Alabi *et al*., 2020). This may be attributed to phytochemical compounds that have antimicrobial activity (Bukar *et al*., 2010), such as Saponins that can smooth the digestive system and kill harmful microbes and tannins that have antiseptic properties having good effect on the digestive tract (Dewi *et al*, 2014) resulting in easy digestion, optimal nutrient absorption and better body weight (Astuti and Suripta, 2020). On the other hand, Ma'rifah *et al*. (2023) documented that *Moringa Oleifera* leaf extract (MOLE) impaired body weight, body weight gain and feed conversion ratio of broilers ($p<0.05$). Additionally, Alabi *et al*. (2017) and Paul *et al*. (2018) reported that (MOLE) reduced feed intake and improved feed efficiency in broiler. However, results of the present study showed no significant effect on broiler performance at both densities in harmony with that detected

Table 5. Effect of Moringa aqueous extract and synbiotic supplementation on liver pathology of broiler chickens reared at two different densities.

		Fatty change	Bile duct hyperplasia	Granulomatous hepatitis	Total histopathological score (THPS)
CD10		1	1	2	1.33
MAECD10		0	0	0	0
SynD10		0	1	1	0.67
CD15		2	3	2	2.33
MAECD15		0	1	1	0.67
SynD15		0	1	0	0.33
	SD	NS	*	NS	NS
Significance	D10.ttt	NS	NS	NS	*
	D15.ttt	**	NS	NS	**

Results were expressed as means ± standard error,*,** indicate significance within columns($P<0.05$ and 0.01 respectively). CD10: Control group density 10 birds/m^2, MAED10: Moringa aqueous extract density 10 birds/m^2, SynbD10: synbiotic density 10 birds/m^2, CD15: control group density 15 birds/m^2, MAED15: Moringa aqueous extract density 15 birds/m^2, SynbD15: Synbiotic density 15 birds/m^2, SD: Stocking density, D10.ttt: 10 birds/m^2 treatment, D15.ttt: 15 birds/m^2 treatment, NS: Nonsignificant.

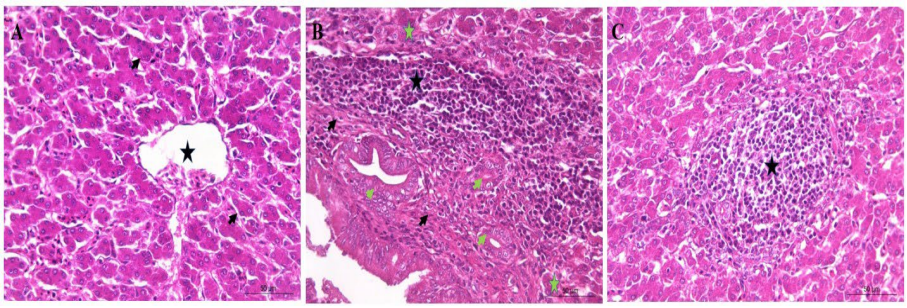

Fig. 2. Moringa aqueous extract and synbiotic supplementation on liver pathology. A) A representative photomicrograph of the normal liver shows the normal architecture of the hepatic parenchyma, normal central vein (star), and hepatocytes in cords are present (arrows) Bar=50µm. H&E. B) A representative photomicrograph of a liver of overstocking density group shows hyperplasia of the bile ducts (green arrows), severe lymphoplasmacytic infiltrations, peritubular (black stars), fibrous connective tissue proliferation (black arrows) and necrosis of the neighbouring hepatocytes (green stars). Bar=50µm. H&E. C) A representative photomicrograph of a liver of overstocking density group shows granulomatous inflammation and necrosis of the neighbouring hepatocytes. Bar=50µm. H&E.

by Khan *et al*. (2022) who demonstrated that MA had no significant effect on feed intake, BW, FCR.

Synbiotic can be safely used to improve growth performance (Dev *et al*., 2020) and Nisar *et al.* (2021), body weight (Hu *et al*., 2022) and feed conversion ratio (Brugaletta *et al*., 2020). Conversely, results of the current study reported non-significant improvement in the growth performance at both densities in agreement with Tavaniello *et al*. (2023) and Cason *et al*. (2023) who noticed no significant alteration of overall trial period growth performance but decreased the 0 to 35 d FCR by 11 points (Cason *et al*., 2023).

The carcass performance is a crucial economic factor of the broiler business (Nasr *et al*., 2019). Carcass yield is important for determining the growth performance and economic returns of broiler meat (Egbu *et al*., 2022). Increasing SD was found to dramatically decrease dressing in the current study, similar findings were reported by Nasr *et al*. (2021). Besides Thema *et al*. (2022) reported that the lowest carcass yield was obtained with SD 15 bird/m^2. On the other hand, our data does not support the concept of Abo Alqassem *et al*. (2018) who found that increasing SD decreased dressing percent but the difference was not significant.

Meat PH is an essential factor for meat stability A high pH can promote the growth of microorganisms, which can lead to rapid spoilage of meat (Aberle *et al*., 2001). However, in our study, there was no difference in relation to the stocking density that agreed with Son *et al*. (2022).

Drip loss is the manifestation of the leakage of myofibers and loss of water, iron, and proteins during the transition of muscle to meat (Ponsuksili *et al*., 2008). Drip loss is very important for palatability, and thus the overall quality and acceptability of meat, and has been a problem for the meat industry, in particular pork and chicken, for many years (Forrest *et al*., 2000). The amount of cooking loss can describe the potential for loss of nutritional value of meat during the cooking process. The low cooking loss value of broiler meat can indicate good meat quality, so to maintain the quality of the meat, various preservation efforts are carried out (Sari *et al*., 2021). Present findings reported an increase in the CL and DL with increasing density that were confirmed by Nasr *et al*. (2021). On the other side, Son *et al*. (2022) and Thema *et al*. (2022) revealed that increasing density achieved no significant change in CL. Similarly, Son *et al*. (2022) reported no change in CL with increasing density.

Water-holding capacity (WHC) of meat and meat products plays an essential part in determining the ultimate weight of the meat (Egbu *et al*., 2022) and determines the visual acceptability, weight loss, cook yield as well as sensory traits upon consumption (Warner, 2023). On the contrary to our findings, there is no significant change in WHC with increasing density (Thema *et al*., 2022). While Son *et al*. (2022) reported an increase in WHC with increasing density.

Nutrients present in *M. Oleifera* such as carbohydrates, protein and fibers with low fat, phenol and flavonoids (Saini *et al*., 2014) can be helpful in improving carcass yield and dressing percent as previously mentioned by Khan *et al*. (2022) and Egbu *et al*. (2022) by using MAE and MSE respectively. On the other hand, results by Astuti and Suripta (2020) using Moringa extracts in drinking showed no effect on the percentage of carcass that also corroborate our findings.

It was not possible in this study to confirm reports assuming that MAE increased meat pH as previously mentioned by Egbu *et al*. (2022). The decreased CL in the present study suggested getting more tender meat when MAE was supplemented. These finding supported by studies of Alabi *et al*. (2020); Egbu *et al*. (2022) and Jiang *et al*. (2023) when they used MAE, MSE and fermented *Moringa Oleifera* successively and confirmed getting more tender meat. Even though, Egbu *et al*. (2022) recorded no alteration in DL & CL when MAE was used.

Carcass characteristics weren't significantly affected by synbiotic supplementation in term of pH (Nisar *et al*., 2021; Tavaniello *et al*., 2023), dressing percent (Sarangi *et al*., 2016; Nisar *et al*., 2021) and WHC (Nisar *et al*., 2021), such findings are similar to those obtained in the present study. However, Dev *et al*. (2020) reported that synbiotic supplementation lower meat PH and increase WHC of broiler meat. Furthermore, dietary synbiotic supplementation was effective in ameliorating compromised meat quality in broilers under cyclic HS by reducing CL and DL (Chen *et al*., 2021), this support findings of current study in that record an improvement of such parameters when Sentobact® was supplemented to HD group.

Corticosterone has been used as a stress indicator (Weina *et al*., 2018) and its secretion leads to mental illness and mood disorders in experimental animals (Iñiguez *et al*, 2018). HSD resulted in increased level of corticosterone levels that was in in accordance with Son *et al*. (2022) who found that corticosterone levels were significantly higher with increasing density. This may be attributed to high fear levels in highly stocked broilers as previously announced by Ghareeb *et al*. (2014) who claimed that The release of CORT is correlated with the development of fearfulness in animals in response to various internal and external stimulations or may be due to increased dopaminergic and serotonergic activities that participate in stimulating cortisol synthesis and its release into the blood (López-Patiño *et al*., 2013; López-Patiño 2021). It also may be due to increased incidence and severity of CD (Yardimci and Kenar, 2008) associated with deteriorated litter quality at HSD (Dawkins, *et al*., 2004).

Moringa Oleifera aqueous extract found to exhibit a hypocortisolemic in highly stocked broilers in our contemporary results similar to those achieved by Shourbela*et al*. (2020) in hypoxic stressed tilapia. Similarly, *Moringa Oleifera* aqueous seed extracts was helpful in minimizing the adverse effects of heat stress in New Zealand white female rabbits by decreasing cortisol levels mainly due to a large variability of its bioactive compounds as declared by Mutwedu *et al*. (2022). On the contrary, Purwoningsih *et al*. (2022) noticed no significant effect of Moringa oil on serum cortisol level of Water-immersion Restraint Stress Mouse.

Synbiotic supplementation had no effect on corticosterone levels (Mohammed *et al*., 2021). Similarly, Cengiz *et al*. (2015) reported that dietary probiotic did not affect CORT concentrations in broilers reared at different stocking densities. The absence of a treatment effect on the plasma CORT concentrations in our study could be attributed to the characteristics of stressors and types of synbiotic or probiotic (Scanes *et al*., 2016).

Monoamines including noradrenaline (NA), dopamine (DA), and serotonin (5 hydroxytryptamine, 5HT) participated in the physiological responses to stress (Øverli, *et al*. 2001). Stress increased dopaminergic and serotonergic activities within the central nervous system (Herrera *et al*., 2020; López-Patiño 2021), the increased brain serotonergic activity is apparently involved in the HSC axis activation, leading to an increased release of A and NA into the blood (Gesto *et al*., 2014). This was consistent with our results that revealed a significant increase in NE, DA and SE with increasing density. Similarly, López-Patiño *et al*. (2021) recorded an increase in brain DA and 5HT in fish submitted to a HSD but not for the NE.

On the other hand, El-Tarabanya *et al*. (2019) reported lowered brain serotonin and DA in rabbits reared with a limited space allowance. Additionally, Ahmed-Farid *et al*. (2021) and Salah *et al*. (2021) recorded lowered SE levels in heat stressed broilers. The altered contents of monoaminergic neurotransmitters dependent on species difference, type of stressor, the duration of exposure to stress that clearly control the response intensity as well as the brain area or tissue assessed (López-Patiño *et al*., 2021).

Researches proved that leaves extract reestablishes levels of monoamine in the brain (Pareek *et al*., 2023), it modulated the alteration induced in neurotransmitter level that elicited by stress due to their role in restoration of brain cellular structure (Mohamed *et al*., 2019) or as a result of alkaloids, terpenes, flavonoids, and sterols (Bhattacharya *et al*., 2018) interacting on the γ-aminobutyric acid subtype A receptors (GABAA-receptors) and activating the noradrenergic, serotonergic, and dopaminergic neurotransmitter systems (García-Ríos *et al*., 2020). This explains the current findings and those mentioned by Mohamed *et al*. (2019) on hypoxic mice after cobalt treatment.

Tryptophan is an essential amino acid for serotonin synthesis and acts directly as an important determinant of mood (Kikuchi *et al*., 2021). Synbiotic supplementation exert an influence on tryptophan metabolism (Hu *et al*., 2018) resulting establishing brain serotonin concentration via the microbiota-gut-brain axis (Gheorghe *et al*., 2019; Mohammed *et al*., 2021), this explains the slight increase in the SE levels with synbiotic supplementation among ND gps. however, synbiotic supplementation resulted in a significant reversing of the high SD. adverse effect. Similarly, Gu *et al*. (2020) announced that Probiotics *Lactobacillus casei* (*L. casei*), reversed the stress- induced expression changes of monoamines could significantly protect against depression in rats. Even though, results obtained by Morshedi *et al*. (2020) announced that the synbiotic in the diabetic rats could increase serotonin level. Moreover, Yan *et al*. (2020) declared that no significant effect was achieved by probiotic supplementation on the concentrations of peripheral and central serotonin and catecholamines as well as their metabolites.

In the present study, highly stocked broilers were more fearful indicated by longer TI durations than broilers housed at lower densities. Past research suggested that housing broilers at high stocking densities can contribute to increased fearfulness (De Jong *et al*., 2012). On the contrary, Anderson *et al*. (2021) found that birds housed at higher stocking densities showed reduced TI durations, suggesting reduced fearfulness.

Anxiety results from an imbalance or abnormal functioning of excitatory neurotransmitters systems such as serotonin, norepinephrine, dopamine and glutamate; or the gamma-amino butyric acid (GABA), an inhibitory neurotransmitter (Kaur & Singh 2017) and fear is a major psychological feature that accompany anxiety (Hendriks *et al*., 2016).

The anxiolytic-like effect of *Moringa Oleifera* leaves Aqueous extract that was obvious in HD treated group in the our recent study may be referred to a large amount of alkaloids, terpenes, flavonoid and sterols in *Moringa Oleifera* leaves (Bhattacharya *et al*., 2018) that is known to have antidepressant anxiolytic effect effects by interacting on the γ-aminobu-

tyric acid subtype A receptors (GABAA-receptors) and by activating the noradrenergic, serotonergic, and dopaminergic neurotransmitter systems (García-Ríos *et al*., 2020; Pratiwi *et al*., 2023) and the presence of alkaloid, triterpenoid, flavonoid, and carbohydrate that antagonize each of restlessness, irritability, and fearfulness in mice (Aburawi *et al*., 2021). Similar results obtained by de Siqueira Patriota *et al*. (2023) who found that the water-soluble lectin from *Moringa Oleifera* seeds (WSMoL) exhibits an anxiolytic-like effect in mice and confirmed the claim of Noubissi *et al*. (2022); Pratiwi *et al*. (2023) who Emphasized that *Moringa Oleifera* leaf-aqueous extract have anxiolytic actions.

Despite the absence of synbiotic supplementation effect on TI at normal density but it was obvious at high density group. such findings confirmed by those of Mohammed *et al*. (2021) who claimed that the supplement improves the fear state and related stress response in broiler chickens under HS conditions. The improved fear responses with synbiotic supplementation may be attributed to its functions in producing neuroactive substances affecting brain neural signaling via the enteric nervous system, the vagal afferents, and/or the bloodstream (Valles-Colomer *et al*., 2019).

The liver is an important metabolic and immune organ, through histopathological examination it was found that increasing SD resulted in increased inflammation of liver which is consistent with the research results of Xin *et al*. (2022) who found that HSD group had more inflammatory cells, hepatocyte edema, and the total histopathological score significantly increased. It was reported that HSD reduced the antioxidant capacity of the serum and tissue of broilers (Miao *et al*., 2021) causing oxidative stress, accelerating tissue oxidative damages (Ikwegbue *et al*., 2017), reducing immunity and causing liver damage (Gholami *et al*., 2020).

MA supplementation decreased liver fatty change and alleviated liver damage in HD group. This was previously noticed by Ibrahim *et al*. (2022) who declared that the aqueous extract of *M. Oleifera* has a great potential to prevent and improve liver damage in rat. Moreover, *Moringa Oleifera* leaves extract was found to reverse the severity of hepatotoxicity induced gentamicin in broilers (Arafat *et al*., 2018). On the other hand, Alabi *et al*. (2020) found that liver hypertrophy, hyperplasia, inflammation, necrosis and injury were not affected in broiler liver by MA supplementation.

The improvement in liver health and total histopathological score seen in the current study with synbiotic supplementation especially in high density group was also announced by Hashem & Mohamed (2009) who found that synbiotic decreased liver inflammation, improved liver health and was effective in alleviating toxic effect of aflatoxins in broiler ration. Additionally, a study by Cai *et al*. (2023) revealed that synbiotic supplementation can improve liver function and reduce the degree of liver fibrosis.

It is worth mentioning that the controversy in results among the published literature could be due to the dose, duration, extraction method, broiler strain and other experimental protocols (Khan *et al*., 2022).

Conclusion

In the light of previously mentioned and discussed findings, it is worth mentioning that Increasing stocking density from 10 to15 birds/ m^2 adversely affect broilers BWG, some carcass quality parameters, increased corticosterone, fear levels, alter brain monoamines levels and deteriorate liver health. MAE and Synbiotic supplementation caused a non-significant improvement in performance but significantly improve some carcass quality parameters, decreased stress and fear levels, reverse the brain monoamines imbalance improved liver health but the effect was more obvious at HD.

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Conflict of interest

All authors declare that they have no known competing financial in¬terests or personal relationships that could have appeared to influence the work reported in this paper.

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