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EFFECT OF DIETARY HERBAL EXTRACT AND PROBIOTIC SUPPLEMENTATION ALONE OR IN COMBINATION ON GROWTH PERFORMANCE, MEAT QUALITY, AND STRESS INDICATORS OF BROILERS SUBJECTED TO HIGH STOCKING DENSITY

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Ph. D. THESIS

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ABBREVIATIONS

BWG **:** Body weight gain

CAT **:** Catalase

H:L ratio **:** Heterophil to lymphocyte ratio

HSD **:** High stocking density

MDA **:** Malondialdehyde

O^{2-} **:** Superoxide ion

SOD **:** Superoxide dismutase

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ÖZET

YÜKSEK YERLEŞİM SIKLIĞINDA YETİŞTİRİLEN ETLİK PİLİÇLERİN RASYONLARINA BİTKİSEL EKSTRAKT VE/VEYA PROBIYOTİK İLAVESİNİN BÜYÜME PERFORMANSI, ET KALİTESİ VE STRES DEĞİŞKENLERİ ÜZERİNE ETKİLERİ

RAZA I. Aydın Adnan Menderes Üniversitesi, Sağlık Bilimleri Enstitüsü, Hayvan Besleme ve Beslenme Hastalıkları Programı, Doktora Tezi, Aydın, 2021.

Amaç: Bu çalışmada, kavun-SOD ve/veya *Pediococcus acidilactici*'nin yüksek yerleşim sıklığında barındırılan etlik piliçlerde büyüme performansı, et kalitesi ve stres göstergeleri üzerine etkileri araştırıldı.

Gereç ve Yöntem: Çalışmada 504 adet bir günlük yaştaki erkek etlik civciv, 6 tekrar grubu içeren 5 deneme grubuna rastgele dağıtılmıştır. Tekrar grupları 1 m^2'lik yer bölmelerinde barındırıldı. Toplam 42 gün süren araştırmada bazal rasyonlar hazırlandı. Kontrol grubu normal yerleşim sıklığında (12 tavuk/m^2) barındırılmış ve bazal rasyon ile beslendi. Diğer gruplar yüksek yerleşim sıklığında (18 tavuk/m^2) barındırıldı. Yüksek yerleşim sıklığında yetiştirilen gruplardan biri (HSD) bazal rasyon ile beslenirken diğerlerinin (kavun-SOD, *P. acidilactici* ve kavun-SOD + *P. acidilactici*) rasyonlarına sırası ile kavun-SOD (12,5 mg/kg), *P. acidilactici* (100 mg/kg) ya da bunların kombinasyonu ilave edildi.

Bulgular: Büyüme performansı, et kalitesi ve H:L oranı bakımından gruplar arasında fark oluşmadı. HSD grubunda serum SOD düzeyi kontrol, kavun-SOD ve kavun-SOD + *P. acidilactici* gruplarına kıyasla daha düşük (P = 0,046) bulundu. Serum MDA yoğunluğu kavun-SOD ve *P. acidilactici* gruplarında HSD grubuna göre daha düşük (P = 0,050) olarak belirlendi. Göğüs eti SOD aktivitesi bakımından gruplar arasında herhangi bir değişiklik oluşmadı. HSD grubunda göğüs eti MDA düzeyinin diğer gruplara kıyasla daha yüksek (P = 0,009) olduğu belirlendi.

Sonuç: Bu çalışmada kavun-SOD ve *P. acidilactici* HSD kaynaklı oksidatif stres azaltığı belirlendi.

Anahtar kelimeler: Bitkisel ekstrakt, Et kalitesi, Etlik piliç, Performans, Probiyotik.

ABSTRACT

EFFECT OF DIETARY HERBAL EXTRACT AND PROBIOTIC SUPPLEMENTATION ALONE OR IN COMBINATION ON GROWTH PERFORMANCE, MEAT QUALITY, AND STRESS INDICATORS OF BROILERS SUBJECTED TO HIGH STOCKING DENSITY

Raza I. Animal Nutrition and Nutritional Diseases Program, Graduate School of Health Sciences, Aydın Adnan Menderes University, Ph. D. Thesis, Aydın, 2021.

Objective: Effect of dietary melon-SOD and/or *Pediococcus acidilactici* on growth performance, meat quality, and stress indicators of broilers subjected to high stocking density (HSD) were investigated in this study.

Material and Methods: A total of 504 one-day-old Ross 308 male broiler chicks were randomly distributed to 5 groups, each consisting of 6 replicate pens, each pen measuring 1 m^2. Basal diets were formulated. One group was not subjected to HSD, fed diets without supplementation, and served as the control group (12 chicks/m^2) whereas the remaining 4 groups were reared at HSD (18 chicks/m^2). One of these HSD groups was fed basal diets without any supplementation. Other HSD groups received dietary melon-SOD (12.5 mg/kg), *P. acidilactici* (100 mg/kg), or a combination of both.

Results: Growth performance, meat quality, and H:L ratio were not different among the treatments. Serum SOD activity decreased in HSD group compared to control, melon-SOD, and melon-SOD + *P. acidilactici* groups (P = 0.046). Birds fed melon-SOD or *P. acidilactici* alone had lower serum MDA levels than those reared at HSD (P = 0.050). SOD activity in breast meat remained unaffected. However, MDA levels in breast meat were greater in broiler chickens in the HSD group in comparison with other groups (P = 0.009).

Conclusion: In this study, melon-SOD and P. acidilactici reduced the HSD-induced oxidative stress in broilers.

Keywords: Broilers, Herbal extract, Meat quality, Performance, Probiotic.

1. INTRODUCTION

Broiler farming has been recognized as a profitable enterprise than ruminants as it becomes ready for human consumption within a very short period. The profitable and better performance of broiler production is influenced by factors like environment, nutrition, management, and genetic makeup. The stocking densities of broiler birds differ between breed, countries, and production systems. Increased stocking density may give higher profitability per kilogram chicken if the performance of birds remains constant (Feddes et al., 2002). For many years, stocking density was defined as the number of birds being raised in provided housing areas (Rice and Botsford, 1925). As broiler production was getting so commercial then the target for the producers changed to the final broiler body weight. Therefore, the stocking density has been is redefined as mass per unit of space, calculated as body mass (in kg or lb.) per unit of housing space (in m^2 or ft^2) (Thaxton et al, 2006). Flocking conditions (up to 22 birds/m^2) may cause oxidative stress leading to increased glutathione peroxidase concentrations in serum (Simsek et al., 2009). At high stocking density (HSD), the bird's performance is affected by restricted airflow and high temperature (Feddes et al., 2002).

It has been stated that oxidative stress is induced by the production of reactive oxygen substances under high environmental temperatures (Zhang et al., 2013). High ambient temperature and HSD can lead to oxidative disruption at the cellular level and cause oxidative stress (Sohail et al., 2011). Studies have revealed that the addition of antioxidants in feed reduces the oxidative stress effects that lead to increased consumption of feed by chicks (Wang et al., 2008). Yasui and Baba (2006) explained that when oxidative stress occurs, superoxide dismutase (SOD) degrades the superoxide ions (O^{2-}) into oxygen and hydrogen peroxide that is later broken down by glutathione peroxidase or catalase. Hence, SOD also has a pro-oxidant effect. SOD may attain from several sources but its poor bioavailability is the main concern and needs developments (Vouldoukis et al., 2004). Melon-SOD is a melon concentrate rich in SOD that is gastro-resistant. Usually, it is well noticeable that plants derived products may exhibit health benefits with fewer side effects as compared to conventional medicine.

In broiler nutrition, probiotics give a functional nutritional line targeting gut health and provide a good substitute to antimicrobial growth promoters (Zulkifli et al. 2000; Applegate et

al. 2010). Under stressful conditions, probiotics can establish and maintain a favorable microbial population in the digestive tract, subsequently improve the assimilation of the food particle and performance (Panda et al. 2000). Supplementation of probiotics in poultry diet may influence their health and performance by alteration in gastrointestinal microflora (Netherwood et al., 1999). *Pedicoccus acidilactici* is a probiotic bacteria that play a positive role in the balance of intestinal flora, and also strengthens the immune response thus leads to better animal performance (Vittorio et al, 2005). The additive Bactocell® is a preparation based on a strain of *P. acidilactici* CNCM I-4622 that can be used in poultry.

The purpose of this study was to evaluate the impact of dietary melon-SOD and a single strain probiotic *P. acidilctici* alone or in combination on growth performance, meat quality, and stress indicators of broilers subjected to HSD.

2. LITERATURE REVIEW

2.1. Stocking Density

In recent times, increased demand for white meat that is low in cholesterol, leaner, and a good source of nutrients and protein has increased the number of poultry farms (Vargas-Rodriguez, et al., 2013). In the last decades, the poultry industry has risen as a profitable business as it produces good quality meat with the least production cost (Simitzis et al., 2012). Stocking density is an important environmental factor in broiler production as it has effects on broiler health, welfare, and well-being, as well as on performance (Estevez, 2007). However, Shanawany (1988) explained that high stocking densities decrease the production cost and produce more kilograms of meat per area, hence, up to an optimum point, profitability increases with HSD.

The stocking density is defined as "body mass (kg) or a number of birds per unit of housing space (m^2)". Researches have revealed that at HSD male broilers (2.7 kg) showed more adverse responses as decreased final body weight, FCR, and performance (Bilgili and Hess, 1995) as compared to the female broilers (2.2 kg) (Puron et al., 1995). This difference perhaps relates to the body mass of the final body weight of birds (Bilgili and Hess, 1995). Accordingly, instead of a number of birds, the body mass of the birds is affecting more to the performance of the bird. Therefore, stocking density is defined as body weight per floor space more accurately for broilers (Bilgili and Hess, 1995; Feddes et al., 2002). According to the European Commission (2007), the maximum permissible stocking density is 33 kg/m^2. Though, a higher stocking density (up to 42 $kg/m^{2)}$ may be authorized if certain standards such as concentrations of NH_3 and CO_2 within the shed, temperature, humidity, and mortality rate are met. The better growth rate with least production price and space allowance is achieved at the cost of a decrease in bird's movement which most of the time result in spending more time sitting (Buijs et al., 2009). The ultimate goal of poultry producers is to increase the economic profit by increasing the body weight of the bird in a unit of floor area along with the least production losses at high stocking density (Rashidi et al., 2018).

2.1.1. High Stocking Density and Growth Performance

Many studies are done to analyze the effect of stocking density (20 to 40 kg/m^2) on broiler production and performance, though most of these studies were unable to give a comprehensive conclusion (Abudabos et al., 2013). According to some researches, there are large benefits on broilers performance by reducing stocking density (Dozier et al., 2005, 2006; Škrbic et al., 2009), on the contrary , some studies showed that decreasing the stocking density had little effect on growth performance. (Thomas et al., 2004, Feddes et al., 2002). Abudabos et al. (2013) documented that high stocking density (40.0 kg/$m^{2)}$ at the finisher stage can be suggested for cost-effective broiler production, however, it may not comply with the poultry welfare criteria. To achieve higher growth relative to the genetic potential of birds, optimum temperature and other environmental conditions should be maintained (Cangar et al., 2008). Decreased stocking density rate has a positive influence on weight gain, feed intake (FI), and feed conversion ratio (FCR) of broilers at starter period but these effects disappear at the finisher period (Abudabos et al., 2013). In contrast, some researchers found that stocking density has no significant impact on body weight gain (BWG) at different rearing stages (Buijs et al., 2009; Housmand et al., 2012). The researcher also explained that during the starter rearing period, the small-sized birds have more space for movement as compared to later stages (grower and finisher) when there is restricted movement and limited availability of the water and feed (Cengiz et al., 2015). The decreased FI at HSD is well understood due to less space allowance for the mobility and capability of bird's body postures to access feed at the lesser feeder space within the pen (Beg et al., 2011).

In hot environments, decreased performance at HSD is caused by the increase in heat production that leads to the reduced air flow at bird level and the bird can dissipate less amount body heat to the environment (Cengiz et al. 2015). Estevez (2007) explained that when there is reduced airflow around the bird, feed consumption and weight gain are lessened which results in high FCR. Bird's performance is affected by many factors like low access to feed and water, high ammonia level, and poor air quality (Houshmand et al., 2012). The factors that affect the performance of birds negatively are limited access to water and feed, poor air quality, and increased ammonia because of inadequate air exchange at the bird level (Chegini et al., 2019).

Several studies have revealed that the incidence and abundance of digestive pathogens can be increased by HSD (Dahiya et al., 2006). High stocking density changes the bird's microflora of digestive track and decreases the overall growth performance (Guardia et al., 2011). The HSD may increase the dust and population of airborne bacteria (Sauter et al., 1981) thus damaging the bird's health directly either by ingestion of contaminated feed or altering the immune responses (Lai et al., 2009). Due to HSD, competition for feed and water increases litter moisture, elevates ammonia level due to degradation of uric acid by the microorganisms which causes high mortality, greater incidence of leg problems and contamination of carcass in broilers (Jayalakshmi et al., 2009). Rashidi et al., (2018) reported that stocking density up to 18 birds/m^2 of floor space cause low BWG in the grower period and also had a deleterious effect on fecal moisture levels that creates walking disorders and poor footpad and hock scores. The footpad score was changed and affected due to poor litter quality when stocking density was about 40 and 45 kg of body weight/m^2 of floor space (Dozier et al., 2005). Although, HSD increased litter moisture (Jayalakshmi et al., 2009) and high ammonia levels made the litter cakey but there was no significant effect on birds' gait and movement (Dozier et al., 2005). But in another study, the bird's gait score 4 and 5 was reported with space allowance 0.0625 m^2/bird (Sørensen et al., 2000). At HSD, worse GS is correlated with the movement of birds (Estevez et al., 1997; Andrews et al., 1997) and poor litter quality (Wang et al., 1998) alone or in combination. The experimental results of Guardia et al. (2011) showed no abnormality in the locomotive behavior of birds, although the litter quality was poor because of HSD.

Elfadil et al. (1996) said that HSD results in scratches on the whole carcass but (Dozier et al., 2005) observed increased scratches on the thigh and back areas with no occurrence of tears. Feddes et al., (2002) in their study found that HSD has a non-significant effect on the carcass grade with no scratches on the skin were observed. HSD does not affect breast muscle yield (Feddes et al., 2002; Bilgili and Hess 1995). Through several studies, it is confirmed that HSD had no direct effect on mortality (Offiong et al., 2001; Feddes et al., 2002; Hadorn et al., 2002; Thomas et al., 2004; Meluzzi et al., 2008; Beg et al., 2011; Guardia et al., 2011).

2.1.2. High Stocking Density, Physiological and Oxidative Stress

Different environmental factors can lead to stress in the poultry industry (Heckert et al., 2002). Moberg (2000) defined stress as "any biological reaction developed by an animal

or bird when its homeostasis is threatened". Among different environmental issues, high stocking density (HSD) has a stress generating effect on bird's immunity and performance (Estevez, 2007). Zulkifli and Azah (2004) explained that birds change their behaviors under stressful conditions, and these new behaviors may increase energy consumption. Physiological stress levels have been measured as a result of HSD (Estevez, 2007). But there is no substantial effect observed for the stress indicators such as plasma (Jones et al., 2005) and fecal corticosterone concentrations (Dawkins et al., 2004; Buijs et al., 2009), plasma glucose and cholesterol concentrations (Thaxton et al., 2006). Another physiological stress indicator is the weight of bursa fabricius and spleen. (Puvadolpirod and Thaxton, 2000b). Weights of lymphoid organs can indicate the birds' immune response and immunity status Pope, 1991). Studies showed that stocking density did not significantly impact spleen and bursa weights (Heckert et al., 2002; Buijs et al., 2009; Housmand et al., 2012; Tong et al., 2012). However, other experiments revealed that bursa weight was decreased by the increase in stocking density (Simitzis et al., 2012; Ravindran et al. (2006). According to Tong et al. (2012), there were no significant changes in immune parameters due to HSD.

During HSD, a state of hemodilution takes place, which modifies the oxygen supply and body temperature, resulting in changes in packed cell volume. High ambient temperature and high stocking densities may cause oxidative stress that can arouse oxidative disruption in nucleic acids, proteins, and lipids (Sohail et al., 2011). Lan et al. (2004) explained that under high environmental temperature, reactive oxygen substances are produced that cause oxidative stress. The stress indicator, heterophil: lymphocyte (H:L) ratio relationship with HSD (Gross and Siegel, 1983), is still debatable as Heckert et al. (2002) did not observe any differences among H:L ratio while Thaxton et al. (2006) observed increased ratio by increasing the stocking density. However, Thaxton et al. (2006) explained that stocking densities, at least from 20 to 55 kg of body weight/m^2, did not any cause major physiological stress in broilers. Researchers assume that high stocking density (HSD) starts the struggle between the birds may cause stress, hence resulting in high levels of circulating glucocorticoid (Ravindran et al., 2006). In the presence of a stressor, the body produces more glucose to maintain homeostasis (Virden and Kidd, 2009) that leads to increased blood glucose (Puvadolpirod and Thaxton, 2000). Cengiz et al. (2015) reported that stocking density had no significant effects on blood malondialdehyde (MDA), serum corticosterone, nitric oxide, and H:L ratio levels in broiler chickens. There was a non-significant effect of HSD on blood glucose, cholesterol, CS, and the H:L ratio (Thaxton et al., 2006; Turkyılmaz et al,

2008; Housmand et al., 2012). The research conducted by Abudabous et al., (2013) showed that serum aspartate aminotransferase concentration was increased as the rate of stocking density was increased that points toward hepatocellular damage. Glutathione is an indicator of oxidative stress that shows the equilibrium pro-oxidant and antioxidant in favor of the pro-oxidant state (Simitizis et al., 2012). At HSD, the decreased concentration of glutathione and GSH: GSSG ratio also represents high levels of oxidative stress in birds (Simitizis et al., 2012).

2.2. Probiotics

The broiler meat has been evolved as not only cheap but also a safer protein source, however, the massive production also confronted challenges i.e. infectious diseases which could be caused by viruses, bacteria, fungi, protozoa, and others (Alkhalf et al., 2010). This directed the use of antibiotics as therapeutic, prophylactic, and growth promotor in the field (Alkhalf et al., 2010). In last decades, the use of antibiotics in meat production is reduced due to their residues in the meat (Menten, 2001). As fear of antibiotic resistance in consumers, the use of antibiotics was banned in the commercial farming of broilers in EU (Regulation EC No 1831/2003). In commercial farming, newly hatched chicks lack in the rapid development of normal microflora in the intestine (Fuller, 1989). At this stage, the chicks are more vulnerable to pathogenic colonization in the intestine. (Alkhalf et al., 2010). In broilers, the probiotics maintain and develop intestinal microflora balance (Jin et al., 1998; Waititu et al., 2014). Patterson and Burkholder (2003) explained that probiotic microorganisms are a suitable alternative to antibiotics in livestock. Probiotic definitions have also evolved, with Fuller (1989) stating probiotics as a live microbial feed supplement that improves gastrointestinal health. The Food and Agriculture Organization and World Health Organization (2001), defined probiotics as : “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Fric (2007) redefined probiotics as live microorganisms that exhibit healthy effects when ingested at a desired amount. As antibiotics have so many harmful effects, probiotics can be utilized against the pathogenic bacteria in the gut (Fuller, 1992). Probiotics provide a good source of growth promotors by maintaining the gut microbial balance and health of the bird (Mountzouris et al., 2007; Applegate et al., 2010). At the early stage of rearing, bird faces many stressors (Panda et al.,

1999), development of the beneficial microbial population in the digestive tract can improve the digestion of feed and bird performance (Panda et al., 2000). Patterson and Burkholder (2003) in their study, explained the detailed probiotics mode of action: 1) secretions such as organic acids, bacteriocins, and hydrogen peroxide are released against the pathogenic bacteria and 2) by the term called competitive exclusion, that is fighting with other bacteria in that specific space.

Haddadin et al. (2001) mentioned the good influence of probiotic's use on the broiler's performance. Probiotics improve the intestinal microflora and develop epithelial cell and enhance the immune function of the bird (Biggs et al., 2007). The most common probiotic species that are used in poultry nutrition are: *Streptococcus, Lactobacillus, Bifidobacterium, Bacillus, Candida, Saccharomyces* and *Aspergillus* (Awad et al., 2009). *Enterococcus, Pediococcus, Bacillus, Lactococcus, Streptococcus* spp, etc. have been used at a lesser degree in poultry production (Lee et al., 2010b; 2015). Probiotics have a major role in regulating triacylglycerol and cholesterol levels (Lin et al., 1989; Taranto et al., 1998).

Some studies stated that the use of probiotics in broilers have a positive effect on BWG and FCR (Joy and Samuel, 1997; Kumprecht and Zobac, 1998; Fritts et al., 2000), however, some scientists found no valuable effects (Maiolino et al., 1992; Choudhury et al., 1998; Panda et al., 1999; Kahraman et al., 2000; Chamani, 2016). But the researches from the last decades showed a positive impact on BWG by the use of probiotics (Djouvinov et al., 2005a; 2005b; Harimurti et al., 2010).

Chamani (2016) reported that the use of probiotics increased the breast meat yield and decreased gizzard weight. As the demand for breast meat is increased in the recent times, the use of probiotics lead to improved breast meat by scientists (Kabir et al., 2004; Ashayerizadeh et al., 2009). The intestinal morphology is significantly improved, i.e. the villus surface area, villus height, number of goblet cells, and crypt depth were ominously improved by the use of probiotics (Chamani, 2016). Increased villus height indicates an increased surface area capable of greater assimilation of available nutrients (Caspary, 1992). Green and Sainsburg (2001) described that probiotics contribute to intestinal microbial balance and improves gut functionality. The probiotics' mode of action can be understood easily that they manage the low gastric pH and the drastic effects of bile salts (Noohi et al., 2016).

Flint and Garner (2009) summarized the objective of the commercial use of probiotics is to increase animal health and growth performance with reduced morbidity and mortality, thus,

to achieve high economic benefits. However, the efficacy of probiotics is determined by the type and amount of bacterial strains added to the feed (Batkowska et al., 2015). Batkowska et al. (2015) explained that the dose of preparation, the concentration of the strain is crucial to obtain the desired results.

2.2.1. *Pedicoccus acidilactici*

Pedicoccus acidilactici is gram-positive cocci homofermentative that can reproduce in a wide variety of temperature, pH and exert antagonism against enteric pathogens, majorly by producing bacteriocins identified as pediocins and lactic acid (Klaenhammer, 1993). Facultative anaerobic cocci, Pediococci belong to the group of lactic acid bacteria (Noohi et al., 2016). Lactic acid bacteria have the properties to enhance the immune system that aids to fight against enteric diseases in poultry (Huang et al., 2004). After the awareness of antibiotic resistance, scientists are proposing that lactic acid bacteria may be useful as an alternative source of antibiotics in poultry production (Messaoudi et al., 2011).

2.2.2. *Pedicoccus acidilactici* and Growth Performance

By maintaining healthy levels of microbiota and reducing infectious diseases, *P. acidilactici* is beneficial (Ooi and Liong, 2010). Fuller (1977) established host-specific Lactobacillus strains, found it can decrease E.coli in the small intestine and crop. *P. acidilactici* improves animal performance by balancing intestinal flora and strengthening the immune system (Vittorio et al, 2005). Many studies have proved that *P. acidilactici* may improve body weight gain, FCR, and meat quality by maintaining the intestinal microflora of the chickens (Djezzar et al., 2012). It was showed by Papagianni and Anastasiadou (2009) that Pediococci release not only lactic acid, but also antimicrobial peptides, such as pediocins, which fight other microorganisms. The use of *P. acidilactici* as a probiotic increased not only BW, FCR, crude protein digestibility but also improved ileum and villus height of jejunum (Mozafarai et al., 2016). The probiotic strain's efficacy in the gut depends upon the resistance against acid and bile salts for having health benefits (Tuomolo et al., 2001). However, in vitro results of *P. acidilactici* showed a good resistance capability counter

to bile salts and acidic conditions (Erkkila and Petaja, 2000; Mathys et al., 2007). Jamila et al (2011) found in their study that *P. acidilactici* restrain the pathogenic colonization of Shigella, Salmonella, Clostridium difficile, and Escherichia coli in the small intestine among small animals. The study done by Habibi et al. (2013) demonstrated that *P. acidilactici* reduced the gut acidity, activated the bacterial enzymes, improved intestinal epithelium status, hence, increased assimilation of nutrients and improved the bird's immunity and growth performance. The supplementation of *P. acidilactici* in broilers feed, improves nutrient intake, glucose and reduces abdominal fat and thus increases the carcass yield (Shabani et al, (2012; Habibi et al., 2013). Habibi et al (2013) found that the weight of the breast was higher following the use of *P. acidilactici* in his experiment.

2.3. Superoxide Dismutase

McCord and Fridovich (1969) discovered the first superoxide dismutase (SOD) in 1969. SOD can be used for the treatment of inflammatory disorders due to its antioxidant effects (Yasui and Baba (2006). Yasui and Baba (2006) explained the SOD mode of action under oxidative stress i.e. SOD degrades the superoxide ions O^2 into oxygen and hydrogen peroxide, which is further degraded by glutathione peroxidase or catalase. It is well acknowledged that superoxide ions O^2 can be eliminated by the antioxidant action of SOD (Abreu and Cabelli, 2010; Fukai and Ushio-Fukai, 2011). Fridovich (1997) expressed the chemical degradation of the superoxide anion ($O_2^{\bullet -}$) in the equation ($2O_2^{\bullet -}+2H^+ \rightarrow H_2O_2+O_2$). That's why, we can say SOD a pro- oxidant because peroxide is produced as the result of O_2 dissociation and further degraded by catalase (CAT) or glutathione peroxidase (GPx) (Fukai and Ushio-Fukai, 2011). Reactive oxygen substances (ROS) are unstable in nature and can damage other elements i.e. proteins, membrane lipids, and DNA (Rice-Evans et al., 1995). After studies, now we have well-defined 3 isoforms of SOD in mammals (Zelko et al., 2002). Depending upon the metallic catalytic ions Mn, Fe, Cu, Zn& Ni, SOD can be classified in the following three classes: 1) Cu, Zn SODs, 2) Mn-SOD/Fe SODs, and 3) Ni SODs (Campbell et al., 1986; Chang et al., 1988; Wan et al., 1994; Jones et al., 1995; Duttaroy et al., 1997; Folz et al., 1997; Blackney et al., 2014). SOD possesses distinct subcellular localization for example in a eukaryotic cell: in the cytoplasm and extracellularly, its Cu/Zn SODs and mitochondria have Mn SODs (Miller, 2012).

There are several sources of SOD, but its poor bioavailability is the subject of study by scientists (Arangoa et al., 2001; Dugas, 2002; Vouldoukis et al., 2004). Superoxide has a short life and cannot pass through cell membranes easily, that's why it acts where it is produced (Wang et al., 2018). Melon concentrate's high antioxidant capacity is due to its high level of SOD connotations (Carillon et al., 2012). Dietary melon supplementation can improve endogenous antioxidant defenses in the target organs, such as the liver (Carillon et al., 2013), adipose tissue (Carillon et al., 2014a), or heart (Carillon et al., 2014b).

SOD that is named chicken SOD was isolated from the liver has divided into two types. Weisiger and Fridovich (1973) wrote that one was present in the mitochondria whereas the other was localized in the cytosol. In poultry, heat stress (34°C) induced a major production of ROS and thus antioxidant enzymes, including SOD, CAT, and glutathione peroxidase (Yang et al., 2010). Peter (2016) explained that in poultry production, SOD has a significant protective role in heat and cold stress, toxicity stress, and other oxidative stress-related conditions.

3. MATERIALS AND METHODS

3.1. Ethical Approval

This study was conducted at the Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Turkey. Before beginning of the experiment, the local ethical committee of the university approved this study on 25th December 2018 (letter No. 64583101/2018/137).

3.2. Experimental Design and Treatments

The design of the study was completely randomized that included a total of 504 broiler male chicks (Ross 308) randomly distributed in 5 groups consisting of 6 replicates each. The control groups were organized based on stocking density as negative control (normal stocking density 12 chicks/m^2) and positive control (HSD; 18 chicks/m^2) fed basal diets without any supplementation. The other 3 experimental groups were raised at HSD fed the basal diet supplemented with melon extract (melon-SOD), or *P. acidilactici*, or a combination of both (melon-SOD + *P. acidilactici*). Melon-SOD is an extract obtained from freeze-dried melon juice concentrate that is rich in superoxide dismutase (SOD 2.6 × 10^6 IU/kg; MELOFEED, Lallemand Animal Nutrition, France) was added to the basal diet at a dose of 12.5 mg/kg (12.5 g/ton). *P. acidilactici* (10 × 10^9 CFU/g; BACTOCELL, Lallemand Animal Nutrition, France), a single strain probiotic, was supplemented at a dose of 100 mg/kg (100 g/ton). Corn soybean-meal based basal diets (Table 1) were formulated for starter (0 to 10 days), grower (11 to 24 days), and finisher (25 to 42 days) according to the nutrient requirements for broilers outlined in NRC (1994).

Table 1. Composition of basal diets

Ingredients	Starter	Grower	Finisher
		%	
Corn	55.63	56.15	59.05
Soybean meal (48% CP)	37.50	36.00	33.50
Vegetable oil	2.50	4.15	4.25
Limestone	0.89	0.85	0.80
Dicalcium phosphate	2.30	2.00	1.73
Salt	0.35	0.35	0.35
DL-Methionine	0.37	0.25	0.12
L-Lysine sulphate	0.21	-	-
Vitamin premix[1]	0.10	0.10	0.10
Mineral premix[2]	0.10	0.10	0.10
Nutrient Composition of Diets, %			
Crude protein	21.75	20.89	19.92
Metabolizable energy (Kcal/kg)	2,910	3,029	3,070
Crude fiber	3.47	3.39	3.30
Crude ash	5.70	5.33	4.95
Calcium	0.97	0.88	0.79
Available phosphorus (Calculated)	0.54	0.48	0.43
Digestible lysine	1.17	1.02	0.96
Digestible methionine	0.66	0.54	0.40

[1] Each kg contained: Vitamin A (as acetate) 12,000.000 IU; vitamin D_3 (as cholecalciferol) 5,000.000 IU; vitamin E (as α-tocoferol acetate 50%) 100,000 mg, vitamin K_3 (as menadione sodium bisulphate 51%) 4,000 mg; vitamin B_1 (as thiamine mononitrate 98%) 3,000 mg; vitamin B2 (as riboflavin 80%) 8,000 mg, niacin (as nicotinic amide 99%) 70,000 mg; vitamin B5 (as calcium D-pentothenate 98%) 20,000 mg; vitamin B6 (as proxidine hydrochloride 99%) 5,000 mg; vitamin B12 (as cobalamin 1%) 30 mg; folic acid (91%) 2,000 mg; vitamin H2 (as D-(+)-biotin 2%) 200 mg; calcium carbonate (as carrier) 63%

[2] Each kg contained: Manganese (as manganese oxide 62%) 150,000 mg; iron (as iron sulphate monohydrate 31%) 120,000 mg; zinc (as zinc oxide 72%) 120,000 mg; copper (as copper sulphate pentahydrate 25%) 12,000 mg; iodine (as calcium iodide 62%) 3,000 mg; selenium (4.5%) 225 mg; molybdenum (as sodium molybdate 39%) 750 mg; calcium carbonate as carrier 13%

3.3. Management

In this study, 12 or 18 chicks/m^2 were placed in each pen (maximum area 110 × 150 cm). Unused areas (feeders, wall ledge) were removed while calculating the density of the settlement. Despite a change of 0.005 m^2/chick, this may develop in the density of the settlements depending on the measurement and the settlement frequency in the partitions. The front panes of the partitions were kept as portable parts.

Standard management procedures were adopted throughout the experiment. During the experiment, feed and water were given as ad libitum. The experiment was continued for 42 days. At 0-10 days of study, chick feeders were used whereas hanging broiler feeders (r = 40 cm, 0.1256 m^2) were used from day 10 and onwards. Wood shavings (8-10 cm deep) were used as bedding material in the research and lighting was provided by daylight and fluorescent bulbs at night. 23L:1D hour lighting plan was applied in the trial.

3.4. Growth Performance

The live weight of birds was recorded on 0, 10, 24, and 42 days. At the beginning and 10th day of the experiment weighing was done by a sensitive scale of ±5 g and weighing on the other days was made with ±10 g scale. Weight gain was determined from the difference between the live weights. Feed intake was also recorded during these growth phases and FCR was calculated.

3.5. Slaughtering Process

At 42 day of the experiment, two birds were randomly selected and slaughtered (12 from each group, 60 in total). The birds were slaughtered by decapitation. Blood samples were collected in serum and heparinized vacutainers for serum separation and estimation of heterophil: lymphocyte ratio (H:L), respectively. After slaughtering, feathers were removed, and birds were eviscerated. Blood samples were allowed to clot for 15 minutes. After clotting, the vacutainers were centrifuged at 4500 rpm for 12 minutes, serum was separated from each

blood sample into the Eppendorf tubes, and stored at -18 ºC until further analysis. Breast meat samples were collected separately for meat quality (100 g) and stress biomarkers (20 g) measurements.

3.6. Meat Quality

Samples of breast meat (approximately 100 g) were collected after the slaughtering. pH and color of breast meat of each broiler chicken were examined 15 minutes after slaughtering and 24 hours after storage at +4 ºC. The probe of the pH meter (Testo 205; Testo Inc., Lenzkirch, Germany) was inserted into the major pectoral muscle. For the color measurements of breast meat, the Minolta CR400 color measurement device, which measures according to the L * (brightness), a * (redness), and b * (yellowness) coordinate system, was used.

Cooking loss was measured after storage at +4 ºC for 24 hours. A known quantity of breast meat it was cooked in a water bath at 80 ºC until the internal temperatures were 75ºC. After cooking, meat samples were cooled, removed from their bags, dried with a paper towel, and weighed again. Cooking loss was calculated as a percentage of the ratio of the difference between the weight of the meat samples before and after cooking to the initial weight (Honikel, 1998).

The water holding capacity of breast meat samples was determined after storage at +4 ºC for 24 hours. A known quantity of breast meat sample was pressed between Whatman's filter paper no. 1 under 2250 g weight (Barton-Gade et al., 1993). The percentage of the ratio of the difference between the weight of the meat sample before and after pressing to the initial weight was subtracted from 100 to calculate the water holding capacity.

3.7. Stress Markers in Serum (MDA, SOD, and Corticosterone) and Blood (H:L)

Serum MDA (malondialdehyde) levels were analyzed by using a spectrophotometric method reported by Yoshioka et al. (1979). Mainly, thiobarbituric acid was made to react by boiling with MDA in the presence of trichloroacetic acid followed by measurement of

absorbance of the color of this reaction at a wavelength of 532 nm in a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan).

Serum SOD activity was measured according to the procedure previously defined by Ohkawa et al. (1979). It was estimated by measuring the inhibition in the reduction of nitro blue tetrazolium with the help of xanthine: xanthin oxidase system as a producer of superoxide followed by measurement of absorbance at a wavelength of 560 nm in a spectrophotometer.

Serum corticosterone levels were assayed using ELISA based commercial kit (Enzo Life Sciences, Inc., NY, US) according to the manufacturer's instruction manual.

To determine the H:L ratio, blood smears of the heparinized blood samples were prepared on glass slides. Smears were stained with May-Grünwald–Giemsa (MGG) stain using Pappenheim's panoptic technique. Afterward, 100 white blood cells (leukocytes) were counted at 100X under the light microscope and H:L was calculated (Gross and Siegel, 1983).

3.8. Stress Markers in Breast Meat (MDA and SOD)

To measure the MDA and SOD activity in the breast meat of broiler chickens, homogenates were prepared. For this purpose, 1 g breast meat sample was homogenized in 1/10 (w/v) 150 mM phosphate buffer solution (pH ~ 7.4) in an ice bath at 2000 rpm of a Teflon-glass stirrer (IKA Overhead Stirrer; IKA-Werke GmbH and Co. KG, Staufen, Germany). Later, the homogenates were thawed at 4 ºC, centrifuged at 7000 × g for 10 minutes (Hettich Zentrifugen, Mikro 200 R, Tuttlingen, Germany), supernatants were separated, and stored at -80ºC (NU 9668E, Nuaire, Japan) until further analysis. The MDA and SOD levels were measured in these homogenates according to the procedure described above for serum analysis.

3.9. Statistical Analysis

Data were analyzed using a computer-based statistical software (SPSS version 22.0, NY, US). Normality was tested using Levene's test followed by logarithmic or square root

transformations in the case of a non-normalized dataset(s). Following one-way analysis of variance, Duncan's test was used as post-hoc test with the assumption of 95% ($P \leq 0.05$) confidence interval for significant differences. Data were expressed as mean ± standard error (pooled).

4. RESULTS

4.1. Growth Performance

Live weight (Table 2), body weight gain (Table 3), and feed intake (Table 4) were not different among the treatments at any phase.

Feed conversion ratio remained unaffected at days 0-10, 24-42, and 0-42 irrespective of the treatments (Table 5). However, the feed conversion ratio decreased in the melon-SOD group compared to other groups ($P < 0.010$) at days 10-24. Feed conversion ratio decreased in broilers in the melon-SOD group compared with control, HSD, and melon-SOD + *P. acidilactici* groups ($P < 0.05$).

Table 2. Live weight (g) of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Day	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
0	46.00	46.18	46.16	46.17	46.16	0.128	0.993
10	213.00	210.02	218.07	223.55	230.66	3.928	0.494
24	941.96	960.37	1001.11	985.86	988.69	12.006	0.555
42	2394.83	2407.87	2472.91	2475.72	2461.14	22.528	0.708

[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

Table 3. Body weight gain (g) of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Days	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
0-10	167.00	163.84	171.91	177.38	184.49	3.933	0.499
10-24	728.96	750.35	783.04	762.31	758.03	8.672	0.413
24-42	1452.88	1447.50	1471.80	1489.86	1472.45	17.126	0.949
0-24	895.96	914.20	954.95	939.70	942.52	12.000	0.557
0-42	2348.83	2361.70	2426.75	2429.56	2414.97	22.523	0.709

[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

Table 4. Feed intake (g) of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Days	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
0-10	266.76	252.17	253.31	268.40	281.57	6.279	0.588
10-24	1036.86	1059.80	1023.10	1055.59	1054.13	13.496	0.916
24-42	2421.17	2446.13	2469.03	2551.59	2488.31	32.605	0.789
0-24	1303.63	1311.96	1276.41	1323.98	1335.70	18.131	0.890
0-42	3724.79	3758.09	3745.43	3875.58	3824.00	44.132	0.830

[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

Table 5. Feed conversion ratio of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Days	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
0-10	1.61	1.54	1.48	1.51	1.54	0.031	0.765
10-24	1.43[a]	1.42[a]	1.31[b]	1.38[a]	1.39[a]	0.012	0.010
24-42	1.68	1.69	1.68	1.71	1.69	0.020	0.988
0-24	1.46[a]	1.44[a]	1.34[b]	1.41[ab]	1.42[a]	0.013	0.028
0-42	1.59	1.59	1.55	1.59	1.58	0.013	0.777

[a, b] Means bearing different superscripts within the same row differ significantly
[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

4.2. Breast Meat Quality

There were no significant differences among the groups for breast meat quality at slaughter and 24-h post-slaughter (Table 6).

Table 6. Breast meat quality of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Item	**Control**	**HSD**[1]	**Melon-SOD**[2]	***P. acidilactici***[3]	**Melon-SOD + *P. acidilactici***[4]	**SEM**	***P*-value**
At slaughter							
L*	51.96	53.37	52.70	53.41	51.77	0.41	0.619
a*	2.78	2.54	2.17	2.25	2.32	0.139	0.650
b*	6.31	6.73	5.89	6.32	5.71	0.231	0.675
pH	6.32	6.12	6.36	6.28	6.25	0.33	0.217
24-h post-slaughter							
L*	61.27	59.43	58.80	58.69	58.69	0.45	0.326
a*	2.81	3.07	2.81	2.63	3.16	0.13	0.730
b*	10.05	10.81	9.40	9.85	9.35	0.24	0.319
pH	5.63	5.55	5.56	5.53	5.50	0.02	0.130
Cooking loss (%)	37.81	36.54	36.05	36.35	35.71	0.33	0.343
Water holding capacity (%)	90.20	90.15	90.56	90.93	90.97	0.34	0.911

[1] HSD = High stocking density; [2] Melon-SOD = Supplemental melon-SOD at high stocking density; [3] *P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4] Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

4.3. Stress Markers in Serum and Breast Meat

Blood H: L ratio and corticosterone, SOD, and MDA levels in serum and breast meat of broilers are presented in Table 7 and Table 8, respectively.

Blood H: L ratio was similar among the groups. Although serum corticosterone levels were not different among the groups, it was numerically higher in HSD group than other groups. Serum SOD levels decreased in HSD group compared to control, melon-SOD, and melon-SOD + *P. acidilactici* groups (P = 0.046). Birds fed melon-SOD or *P. acidilactici* alone had lower serum MDA levels than those reared at HSD (P = 0.050).

Breast meat SOD levels were similar among the groups. However, MDA levels in breast meat were greater in broiler chickens in the HSD group in comparison with other groups (P = 0.009).

Table 7. Serum H:L ratio and corticosterone (ng/mL), superoxide dismutase (SOD; U/mg), and malondialdehyde (MDA; nmol/mg) levels in broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Item	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
H: L ratio	0.29	0.44	0.26	0.41	0.40	0.03	0.093
Corticosterone	3.88	5.67	2.83	3.49	3.27	0.64	0.636
MDA	4.76^{ab}	5.02^{a}	4.39^{b}	4.34^{b}	4.50^{b}	0.08	0.050
SOD	1.96^{a}	1.47^{b}	2.16^{a}	1.81^{ab}	2.03^{a}	0.08	0.046

$^{a, b}$ Means having different superscripts within the same row differ significantly
[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

Table 8. Superoxide dismutase (SOD; U/mg) and malondialdehyde (MDA; nmol/mg) levels in the breast meat of broiler chickens at high stocking density fed melon-SOD and *P. acidilactici* alone or in combination

Item	Control	HSD[1]	Melon-SOD[2]	*P. acidilactici*[3]	Melon-SOD + *P. acidilactici*[4]	SEM	*P*-value
MDA	6.99^{b}	9.35^{a}	6.22^{b}	6.31^{b}	6.70^{b}	0.32	0.009
SOD	4.92	4.83	5.62	5.01	5.06	0.16	0.567

$^{a, b}$ Means having different superscripts within the same row differ significantly
[1]HSD = High stocking density; [2]Melon-SOD = Supplemental melon-SOD at high stocking density; [3]*P. acidilactici* = Supplemental *P. acidilactici* at high stocking density; [4]Melon-SOD + *P. acidilactici* = Supplemental melon-SOD + *P. acidilactici* at high stocking density

5. DISCUSSION

This is the first study investigating the effect of dietary *P. acidilactici*, melon-SOD, and their combination on the growth performance, meat quality, and stress indicators of broiler chickens subjected to HSD. Already, no study has evaluated the use of *P. acidilactici* in stressful conditions like HSD or heat stress. Moreover, the difference of melon-SOD with other plant extracts and/or phytogenics in addition to the absence of any study on the use of melon-SOD in broiler chickens creates difficulty in discussing the results of the present study. This becomes further cumbersome in the case of the combination of *P. acidilactici* and melon-SOD.

5.1. Growth Performance

HSD is a physiological and environmental stressor for poultry. Physically, HSD results in the confinement of birds in a smaller area (as more birds are placed per unit area) thereby restricting the movement of chickens in the pen that reduces the access to the feeders and drinkers. Nonetheless, this might not be the case in the early age of chickens attributed to smaller body sizes and lesser requirement of floor space than those in the finisher phase of life. In addition, increasing physiological stress as the birds grow may modify the behavior of broiler chickens towards feed intake causing a decline in body weight gain and adverse effects on the FCR. Besides physiological stress, HSD also gives rise to environmental stress by disrupting the exchange of gases, dust removal, and heat dissipation within the immediate vicinity (microclimate) of birds (Feddes et al., 2002; Banhazi et al., 2008). HSD also creates a microbial imbalance in the broiler intestine resulting in dysbiosis and histomorphological changes in the gut thus exacerbating the nutrient utilization in broiler chickens (Biswas et al., 1999; Bedford, 2001; Lázaro et al., 2003; Józefiak et al., 2004; Guardia et al., 2011). Consequently, feed intake of broiler chickens declines that cause negative effects on the growth performance. This was confirmed as some researchers reported that HSD may not affect the growth performance during the starter and grower phases, however, it dramatically worsens the growth of broilers in the finisher phase (Guardia et al., 2011; Cengiz et al., 2015) whereas other studies reported that HSD deteriorates the growth performance irrespective of

the growth phases of broiler chickens (Feddes et al., 2002; Dozier et al., 2005; Cengiz et al., 2018). This might not have occurred in the present study as growth performance remained unaffected in the HSD group. Similar results were reported by Vargas-Rodríguez et al. (2013) in response to HSD. Possibly, the discrepancies in results lie in the difference of broiler hybrids used, diet composition, management conditions, and stocking densities.

Probiotics form the base of healthy microbiota in the intestine of broiler chickens where beneficial bacteria dominate while the colonization of enteric pathogens is prevented through competitive exclusion (Waititu et al., 2014). The probiotics favor the growth of lactic acid-producing bacteria such as lactobacilli and bifidobacteria, these bacteria increase the acid production and reduce the pH in the intestine of bacteria (Chambers and Gong, 2011). In this way, the probiotics optimize the gut morphology, enhance nutrient utilization, and promote the growth of broiler chickens. To date, no study is available that describes the effect of melon-SOD and *P. acidilactici* alone or in combination on the growth performance of broiler chickens at HSD. There is no study that describes the use of melon-SOD in broiler nutrition. In this study, the growth performance of broilers at HSD was not affected in response to supplemental single strain probiotic *P. acidilactici* and melon-SOD alone or in combination. Our results are in agreement with those of Ebeid et al. (2019) who reported that dietary supplementation of 0, 200, and 400 mg/kg *Bacillus subtilis* did not affect the growth performance of broiler chickens reared at HSD. Likewise, the use of 200 ppm dietary *B. subtilis*, 500 ppm licorice extract alone or in combination did not affect the growth performance of broiler chickens at HSD (Rashidi et al., 2019). Similarly, the growth performance of broiler chickens at HSD was unresponsive to dietary supplementation of multi-strain probiotics (Vargas-Rodríguez et al., 2013; Cengiz et al., 2015; de Souza et al., 2018).

5.2. Breast Meat Quality

The study revealed that breast meat quality remained unaffected across the groups. Increasing stocking densities from 10 birds/m^2 to 16 birds/m^2 did not affect meat quality of broiler chickens (Moreira et al., 2004). Similarly, Simitzis et al. (2012) reported no effect on breast meat quality in response to HSD. In contrast, Ebeid et al. (2019) reported that dietary supplementation of *B. subtilis* in broiler chickens subjected to HSD increased the color index

of breast meat. No study has reported the effect of melon-SOD alone or in combination with probiotics on breast meat quality of broiler chickens reared under HSD.

It is known that breast meat quality is influenced by the pre-slaughter nutritional and homeostatic status of myofibers that come at play during rigor mortis (Zhao et al., 2012). After slaughter, muscle pH declines in response to the anaerobic glycolytic pathway that produces lactic acid that transforms the muscle to meat (Duclos et al., 2007). Meat quality traits are dictated by meat pH that creates an equilibrium between oppositely charged groups on muscle protein that bind the water thus decreasing protein solubility, water binding capacity of proteins and increased shrinkage of muscle proteins due to attraction of charges. Further shrinkage occurs as the attraction between the opposite charges on the proteins in the proximity takes place concluding the electrostatic repulsion between protein chains (Wismer-Perdersen, 1986; Mir et al., 2017). This increases pale, soft, and exudative meat and vice versa. In the present study, breast meat pH was similar among the groups, therefore, meat quality was not influenced among the treatments.

5.3. Stress Markers in Serum and Breast Meat

HSD is known to pose physiological stress in broilers by altering the micro-environment at the level of birds that changes the homeostatic functions. In response, certain stress indicators are increased in the blood such as blood H:L ratio and corticosterone levels.

Blood H:L ratio was not different among the treatments in the present study. These findings are in agreement with those of Li et al. (2019) who reported that HSD does not affect the H:L ratio of broiler chickens at 35 and 42 days of the experiment. Similarly, the blood H:L ratio in broiler chickens subjected to HSD was unchanged at the 4^{th}, 5^{th}, and 6^{th} weeks of the experiment (Türkyilmaz, 2008). Likewise, Cengiz et al. (2015) reported that the blood H:L ratio was similar in broiler chickens reared at low stocking density, HSD, and those fed dietary probiotics at HSD. In contrast, Simitzis et al. (2012) reported that H:L ratio increased in broiler chickens reared under HSD. Similarly, supplemental synbiotic (mannan oligosaccharides, *B. subtilis, and B. licheniformis*) in broilers subjected to HSD exhibited lowered H:L ratio than those in the HSD group (Kridtayopas et al., 2019). No study is available describing the potential effect of melon-SOD supplementation in broilers at HSD.

Differences in results might be due to the differences in environmental conditions, the composition of diets, and stocking densities.

A numerical increase was noted in the serum corticosterone levels of broilers in the HSD group than other groups. While studies are reporting the absence of any effect on the serum corticosterone levels of broilers at HSD (Dozier et al., 2006; Thaxton et al., 2006; Houshmad et al., 2012; Cengiz et al., 2015), other studies have reported the increased circulatory corticosterone levels in broiler chickens in response to physiological stressors (Puvadolpirod and Thaxton, 2000a; Ismail et al., 2014; Kridtayopas et al., 2019). In agreement with our findings, Cengiz et al. (2015) reported that dietary supplementation of a multistrain probiotic did not affect the serum corticosterone levels of broilers at HSD. On the contrary, a reduction was evident in serum corticosterone of broilers fed dietary synbiotic at HSD (Kridtayopas et al., 2019). The difference in results might be attributed to the difference in environmental conditions, dietary composition, probiotic strain, and stocking densities.

Stressful conditions can cause disturbance in the equilibrium between physiological antioxidant and pro-oxidative systems that initiate the oxidative disruption in nucleic acids, proteins, and lipids that may cause oxidative stress in blood and other body tissues at cellular levels (Sohail et al., 2011). Consequently, an increase in reactive oxygen species and free radicals occurs. SOD is a key enzyme that converts the superoxide ions into oxygen and hydrogen peroxide that is further degraded by glutathione peroxidase and catalase enzymes. During oxidative stress, increased free radicals and reactive oxygen species exhaust the SOD that may be insufficient in the processing of superoxide ions. In our study, HSD decreased the SOD activity in serum whereas *P. acidilactici* and melon-SOD alone or in combination increased the SOD activity. A similar trend of SOD activity was seen in the breast meat of broilers although the differences were numerical only. These findings coincide with those of Li et al. (2019) who reported a decrease in SOD activity in serum in response to HSD in broiler chickens at 35 and 42 days of the experiment. Zhang et al. (2013) reported that HSD reduced the serum SOD activity in broiler chickens compared to low stocking density. Additionally, Magnuson et al. (2020) reported that SOD activity in the breast meat was not affected in broiler chickens placed at HSD compared to those at low stocking density. In contrast, other studies reported no effect of HSD on SOD activity in the blood (Ismail et al., 2014; Cengiz et al., 2015; Jobe et al., 2019). The exact mechanism of modulating the SOD activity by *P. acidilactici* is not yet known. An increase in SOD activity in melon-SOD and melon-SOD + *P. acidilactici* seems to have occurred due to increased bioavailability of SOD

supplied through the melon-SOD. In general, SOD possesses poor bioavailability. Melon-SOD used in this study was gastro-resistant and was bound with wheat-derived glisodin/gliadin protein that increases its intestinal absorption and bioavailability. Since the absorbed SOD directly enters the blood circulation, the SOD levels were meaningfully increased in serum of broiler chickens than breast meat. Therefore, *P. acidilactici* and melon-SOD protected the broiler chickens against oxidative stress by increasing the SOD activity in serum and breast meat.

Subjecting the broilers to HSD increased the serum and breast meat MDA levels whereas dietary melon-SOD and *P. acidilactici* alone or in combination lowered the MDA levels in serum and breast meat of broiler chickens at HSD. Various studies have reported increased serum MDA levels in broiler chickens at HSD (Simsek et al., 2009; Zhang et al., 2013; Ismail et al., 2014; Jobe et al., 2019; Li et al., 2019). In contrast, Cengiz et al. (2015) reported that HSD and dietary supplemental probiotics at HSD did not affect serum MDA levels of broiler chickens. In practice, oxidative damage to lipids (peroxidation) increases the MDA levels in serum and tissues. HSD increased the lipid peroxidation (MDA) in serum and breast meat of broiler chickens whereas *P. acidilactici*, melon-SOD, or a combination of both decreased the HSD-induced lipid peroxidation in serum and breast meat of broiler chickens. The exact mechanisms by which *P. acidilactici* and melon-SOD reduced lipid peroxidation are not known. However, this might be due to the increased oxidative stress that increased the production of reactive oxygen species (free radicals). It is well known that free radicals and reactive oxygen species cause oxidation of the lipids (Imlay, 2003) that might be the reason behind increased MDA levels in serum and breast meat of broiler chickens placed at HSD. SOD, as the first line of defense, starts detoxifying the reactive oxygen species and free radicals by converting them into oxygen and hydrogen peroxide that invites catalase and glutathione peroxidase enzymes for further antioxidant defense. Therefore, increased SOD activity in broiler chickens fed *P. acidilactici* and melon-SOD alone or in combination lowered the MDA levels in serum and breast meat of broiler chickens.

6. CONCLUSIONS AND SUGGESTIONS

Although ammonia and other gases were not measured in the house, this study suggests that a stocking density of 18 birds/m^2 may not affect the growth performance and breast meat quality of broiler chickens through careful management of in-house temperature and ventilation. This was evident from the absence of any difference in biomarkers related to physiological stress like the H:L ratio.

HSD increased the MDA levels and reduced the SOD activity in serum and breast meat of broiler chickens. Supplementation of dietary melon-SOD, *P. acidilactici*, or their combination increased the SOD activity and reduced the MDA levels in serum and breast of broiler chickens. This suggests that melon-SOD and *P. acidilactici* can be used alone or in combination to alleviate the HSD-induced oxidative stress in broiler chickens.

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