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EFFECTS OF DIETARY FERMENTED MEALWORM LARVAE AND STOCKING DENSITY ON MANURE AMMONIA GAS CONCENTRATIONS OF BROILERS

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ABSTRACT

This study was conducted to investigate the effects of the supplementation of defatted mealworm larvae meal fermented with probiotics as a new antibacterial feed additive to the diet of broilers reared under normal- (NSD) and high- (HSD) stocking density on pH value, moisture and nitrogen content, ammonia gas concentration and urease enzyme activity of manure of broilers. A total of 450 one-day-old Ross 308 male broiler chicks were randomly distributed into 6 groups of similar mean weight, each containing 5 replicates. Experimental treatments consisted of a 2 x 3 factorial arrangement with two levels of stocking density (12 birds/m^2 as NSD and 18 birds/m^2 as HSD) and three different mash diets: CONT- a corn-soybean meal-based diet containing no fermented defatted mealworm larvae meal (FDM) (0%); FDMLP- the diet obtained by supplementing DM fermented with *Lactobacillus plantarum* to the CONT diet (0.4%); FDMLB- the diet obtained by supplementing DM fermented with *Lactobacillus brevis* to the CONT diet (0.4%). HSD significantly increased the pH value, moisture content, ammonia gas concentration and urease enzyme activity of manure compared as NSD, but, did not influence its nitrogen content. In addition, the FDMLP and FDMLB diets significantly decreased only the ammonia gas concentration and the urease enzyme activity of manure in broilers when compared to the CONT diet. Interaction between the stocking densities and diets significantly affected only the urease enzyme activity of manure. The results indicate that dietary supplementation of FDMLP and FDMLB as new antibacterial feed additives at the level of 0.4% can reduce ammonia gas concentration and urease enzyme activity of manure of broilers regardless of stocking density.

Keywords: ammonia gas concentration, broiler, fermented mealworm larvae, manure, stocking density, urease enzyme activity

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INTRODUCTION

World population is expected to reach over 9 billion by the year 2050. Together with increasing population, broiler meat consumptions are expected to increase by 173% (Boroojerdi and Rajabzadeh, 2021). Therefore, broiler operations are forced to rear broilers under high stocking density (HSD) to meet ever-increasing demands. They use HSD also to reduce production costs, to get higher carcass yields and ultimately to increase profitability of the broiler operations (Khalil *et al*., 2021). However, HSD results in stress and has negative effects on the balance between the pathogenic bacteria with urease and uricase enzyme activities and beneficial bacteria of the small intestine of broilers, in other words, increase the number of pathogenic bacteria (Cook *et al*., 2011; Kridtayopas *et al*., 2019; Sugiharto, 2022). Consequently, pathogenic bacteria such as *Clostridium* and *Bacillus* spp. with urease and uricase enzyme activities multiply rapidly and colonize on small intestinal mucosa and play an important role in ammonia production (Cook *et al*., 2011).

Manure is a major source of nitrogen (N) pollution in the form of ammonia (NH_3) which is a major aerial pollutant (Jiang *et al.*, 2021). Low digestibility of amino acids and proteins in poultry diets also increase the amount of nitrogen and ammonia gas concentration of manure of poultry (Mahardhika *et al.*, 2019). Uric acid produced from protein and amino acid degradation is a major source of ammonia formation in poultry ceca. Manure uric acid is microbially broken down by the pathogenic bacteria with urease and uricase activities and ammonia gas is released (Liu *et al.*, 2023).

Elevated ammonia gas emissions from intensive poultry operations are a global concerns. Such emissions have negative effects on ecosystem, environment, health of humans and birds (Anderson *et al*., 2020). Ammonia gas emitted into the atmosphere spreads to the environment through prevailing winds and accumulates

on dry and wet surfaces (Wang *et al.*, 2020). Ultimately, it causes acidification of soils and accumulation of N in the form of ammonia in the entire ecosystem, especially in water (Anderson *et al.*, 2020). Moreover, soil nitrifying bacteria convert the stored ammonia into nitrates, thus lower groundwater pH levels and increase drinking water nitrate levels. It also causes eutrophication, acidification and nutrification of groundwater and thus threatens biological diversity, human and bird health and environment (Ayiti *et al.*, 2022). Recent studies indicated that atmospheric ammonia gas emissions from poultry houses also had significant negative effects on global climate change (Al-Kerwi *et al.*, 2022).

Different dietary manipulation strategies are implemented to reduce ammonia gas emissions from intensive broiler operations using ground litter-bed systems and HSD (Anderson *et al.*, 2020). For this purpose, dietary manipulation strategies such as reducing the amount of crude protein of broiler diets, adding essential synthetic amino acids, preparing the diet based on digestible amino acids and adding fiber to diet are applied. Besides these strategies, Adegbeye *et al.* (2019) reported that natural feed additives could improve N metabolism, which will in turn reduce the amount of excreted N causing different forms of environmental pollution. In this sense, antibacterial feed additives like probiotics, prebiotics and synbiotics also are supplemented into the broiler diets (Naseem and King, 2018). It was reported that supplementation of probiotic into broiler diets increased the number of beneficial bacteria and reduced the number of harmful bacteria, thus improved microbial flora of intestines (Zou *et al.*, 2022), reduced small intestine pH (Rehman *et al.*, 2020) and pathogenic bacteria-induced urease enzyme activity (Such *et al.*, 2021), thus suppressed ammonia production of the digestive system and manure-originated ammonia gas concentrations (Eglite *et al.*, 2021).

In addition to these, the use of dried mealworm larvae meal fermented with probiotics has recently been introduced as a new antibacterial-based feed additive into broiler diets (Islam and Yang, 2017). Compared to animal-derived feed ingredients, insects as a novel feed ingredient have several advantages such as being able to convert organic residues into protein more efficiently, needing less space and water and having lower environmental impact and high nutritional values (Boroojerdi and Rajabzadeh, 2021; Lee *et al.*, 2022). Presently, insects such as mealworm larvae (M) is not only considered as a nutrient-rich feedstuff (Kwon *et al.*, 2020) but also as an antibacterial feed additive because of antimicrobial peptides (AMPs) (Benzertiha *et al.*, 2020; Elahi *et al.*, 2022) and chitin (Islam and Yang, 2017) for poultry. Chitin content of M was reported as between 4.30-8.91% (Hong *et al.*, 2020). Chitin in M is partially degraded by the acidic chitinase in the proventriculus and gizzard of chicken to produce chitooligosaccharides, a prebiotic (Lee *et al.*, 2022). However, high chitin levels (> 2.42%) in M may impose negative effects on feed intake and protein availability and thereby worsening growth performance of broilers (Mulyono *et al.*, 2019). Due to the above mentioned reasons, both the reduction of high chitin content and the emergence of antimicrobial components of insects such as M and black soldier fly larvae can be performed by solid-state fermentation (SSF) using specific microorganisms with chitinase enzyme activity that are able to degrade chitin (Mulyono *et al.*, 2019; Luparelli *et al.*, 2022). Among probiotic bacteria species, lactic acid bacteria (LAB) are mostly used for solid-state fermentation of M (Islam and Yang, 2017). SSF makes the possibility of using solid-state fermented M as new antibacterial functional feed additive through improving its nutritional value (Luparelli *et al.*, 2022).

It was hypothesized in the current study that the supplementation of defatted M (DM) subjected to SSF with probiotics into the poultry diets as a new antibacterial feed additive can manipulate the small intestine microflora of broilers in favor of beneficial bacteria, suppress pathogen bacteria with urease and uricase enzyme activity and consequently alleviate the detrimental effects of HSD and reduce manure-induced ammonia gas concentration of broilers operations. Therefore, the present study was conducted to evaluate the effects of the supplementation of DM solid-state fermented with two different probiotics as a new antibacterial feed additive into the diet of broilers reared under normal- and high-stocking densities on manure pH, moisture and nitrogen content, ammonia gas concentration and urease enzyme activity.

MATERIALS AND METHODS

Ethics statement: The research was approved by Tokat Gaziosmanpasa University Local Ethics Committee of Animal Experiments (Process no. 2019-HADYEK-47 and 2020-HADYEK-21). The approval, in accordance with animal welfare regulations, ensures the humane treatment of animals in general, experimental design and data analysis.

Animals, diets and experimental diet: A total of 450 one-day-old Ross 308 male broiler chicks were acquired from a commercial hatchery (Anadolu Ross, Ankara, Turkey). The broiler chicks were weighed, wing-banded and randomly distributed into 6 groups of similar mean weight, each containing 5 replicates. From hatching until 6 week of age, the chicks were kept on floor pens bedded with fresh wood shavings as litter. Broiler house temperature was kept at 32ºC for the first week, 28ºC for the second week and gradually reduced and after 27 days of age, temperature was retained at 21°C. A fluorescent lighting schedule of 23 h light and 1 h dark was used

during the experiment with an average light intensity of 20 lux. The diets in mash form and drinking water were provided *ad libitum*.

Experimental treatments consisted of a 2 x 3 factorial arrangement with two levels of stocking density (12 birds/m^2 as normal stocking density, NSD and 18 birds/m^2 as high stocking density, HSD) (Kridtayopas *et al.*, 2019) and three different mash diets: CONT- a corn-soybean meal-based diet containing no fermented defatted mealworm larvae meal (FDM) (0%); FDMLP- the diet obtained by supplementing DM fermented with *Lactobacillus plantarum* to the CONT diet (0.4%); FDMLB- the diet obtained by supplementing DM fermented with *Lactobacillus brevis* to the CONT diet (0.4%). Each treatment had 5 replicates. FDMLP and FDMLB were supplemented into an amount of corn and the mixture was added to diet. Diets were prepared weekly and stored in airtight containers. The corn-soybean meal-based experimental diets were formulated as isonitrogenous and isoenergetic according to phase feeding practices as the broiler chickens advanced in age and weight, as recommended by the breeder (Ross 308, 2007); the starter phase lasted from day 0 to 10, the grower phase was from day 11 to 28 and the finisher phase was from day 29 to 42. Ingredient composition and nutrition content of the control diet are presented in Table 1.

Table 1. Ingredient composition and nutrition content of the control diet (g/100 g, as-fed basis)

Item	Days		
	0-10	**11-28**	**29-42**
Ingredients			
Corn	57.30	58.99	64.00
Soybean Meal (44.8 % CP)	34.86	31.49	28.39
Fish Meal (65 % CP)	1.51	2.65	-
Vegetable Oil	1.92	3.35	3.82
Dicalcium Phosphate	2.20	1.85	2.10
Limestone	0.87	0.78	0.80
Salt	0.34	0.32	0.36
Vitamin Premix[1]	0.25	0.25	0.25
Trace Mineral Premix[2]	0.10	0.10	0.10
DL-Methionine	0.36	0.22	0.18
L-Lysine	0.22	-	-
L-Threonine	0.07	-	-
Calculated nutrient content			
Dry Matter	90.10	90.10	90.10
Crude Protein	23.00	22.00	19.00
ME (MJ/kg)	12.66	13.19	13.40
Ca	1.00	0.90	0.90
P available	0.50	0.45	0.45
Methionine+Cystine	1.09	0.94	0.80
Lysine	1.44	1.23	1.01
Na	0.16	0.16	0.16
Tryptophan	0.30	0.29	0.25
Threonine	0.93	0.84	0.72

[1] Vitamin premix/kg diet: 12 000 IU vitamin A; 1 500 IU vitamin D_3; 50 mg vitamin E; 5 mg vitamin K_3; 3 mg vitamin B_1; 6 mg vitamin B_2; 5 mg vitamin B_6; 0.03 mg vitamin B_{12}; 25 mg niacin; 12 mg Ca-D-pantothenate; 1 mg folic acid; 0.05 mg D-biotin; 2.5 mg apo-carotenoic acid ester; 400 mg choline chloride

[2] Trace Mineral Premix/kg diet: 80 mg Mn; 60 mg Fe; 60 mg Zn; 5 mg Cu; 0.2 mg Co; 1 mg I; 0.15 mg Se

Mealworm larvae (M) (*Tenebrio molitor* L.) purchased from a commercial supplier in Antalya, Türkiye were grown on organic feed mainly consisting of wheat, wheat bran and carrot, without any contamination of animal origin products based on EC regulation (no 1069/09). The 90-d-old M were not starved before being killed. They were freeze-dried overnight to remove moisture. Around 1 kg of freeze-dried M was ground into the meal using a miller. The M meal obtained was full-fat and produced from the larval stage of yellow meal worms. The crude protein content of M meal in the present study was increased by the chemical defatting process since protein may be utilized as substrates by microorganisms for SSF (Son *et al.*, 2021). Defatting of freeze-dried M meal was performed by soxhlet device under optimized extraction conditions using petroleum

ether to M meal ratio of 3:1 L/Kg, at 60°C for 4 h. After defatting, M meal was dried at 40°C for 3 h. As a result of this process, the high fat content of defatted M meal (DM) was reduced from 23% to 6.6% and its crude protein content was increased from 44% to 76.2%.

Two probiotic bacteria (*Lactobacillus plantarum* and *Lactobacillus brevis*) and *Saccharomyces cerevisiae* (baker's yeast) were used in the SSF of DM with probiotics. *Lactobacillus plantarum* strain and *Lactobacillus brevis* strain were isolated from Çeçil cheese and cheddar cheese, respectively. *Saccharomyces cerevisiae* (baker's yeast) was produced from sugar beet molasses. The probiotics with chitinase activity used in the fermentation were purchased from Neslihan Dikbaş Microorganism Culture Collection at the Agricultural Biotechnology Laboratories of Ataturk University. Chitinase enzyme activity of the purchased probiotics was analyzed in the Agricultural Biotechnology Laboratory of Ataturk University according to the method of Senol *et al*. (2014). According to the results of the analysis, the chitinase enzyme activities in terms of ammonium sulfate precipitation level were 15.00 U/L and 11.36 U/L for *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively. *Saccharomyces cerevisiae* (baker's yeast) was commercially supplied.

The fermentation of DM with two different probiotic bacteria was carried out by modifying the method of Islam and Yang (2017) in Semi-Solid Phase Fermenter (Infors-HT, Labfors AG, Bottmingen, Switzerland) in the laboratory of Isparta University of Applied Sciences, Agricultural Faculty, Department of Animal Science. Distiller's dried grains with solubles (DDGS) and defatted rice bran were used as solid media for probiotic strains during the fermentation process with DM. Before fermentation, DM, DDGS, defatted rice bran and water were autoclaved at 121°C for 15 min for sterilization. And then, the dried DM was ground and used to prepare a mixture of 30% DM, 35% DDGS, 35% defatted rice bran and 80% distilled water for the fermentation process. In the first stage of the fermentation, DDGS, defatted rice bran, DM and distilled water were put into the fermenter and then carbon dioxide was added to create an anaerobic environment inside the fermenter. First, 100 ml of incubated *Lactobacillus plantarum* was added to the solid substrate medium in the fermenter and fermented at 38ºC for 48 h under anaerobic conditions. After 48 h, a second fermentation was performed with 1.0% *Saccharomyces cerevisiae* (baker's yeast) activated for 1 h at 37ºC in 250 ml 0.1% peptone water (10 g yeast + 90 ml peptone water) at 38ºC for another 48 h under anaerobic conditions. *Saccharomyces cerevisiae* during fermentation enhances the viability and growth of lactic acid bacteria (LAB), since it provides some nutrients, such as amino acids and vitamins to LAB (Menezes *et al.*, 2018; Shi *et al.*, 2020). After completion of a total of 96-h fermentation process, the fermented product was dried to less than 15% moisture at 32ºC for 24 h using a drying oven. The same fermentation procedure was performed with *Lactobacillus brevis*. To determine the microbial concentration, 1 g FDMLP or FDMLB was serially diluted with 9 ml of 0.85% sterile saline and thoroughly mixed. The counts of total mesophilic aerobic bacteria were then determined by plating serial 10-fold dilutions in triplicate into Plate Count Agar (PCA) (Merck, Darmstadt/Germany) and incubated at 30ºC for 48 h under aerobic conditions. The numbers of LAB were counted by plating serial 10-fold dilutions in triplicate into De-Man Rogosa and Sharp (MRS) agar (Merck, Darmstadt/Germany) and incubated at 39-40ºC for 5 d under anaerobic conditions. Enumeration of yeast and mold was conducted by plating serial 10-fold dilutions in triplicate into Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Merck, Darmstadt/Germany) and incubated at 25ºC for 5 d under anaerobic conditions. After incubation, microbial colonies were immediately counted and expressed as log10 CFU/g. Nutrient composition and concentrations of microorganisms in DM, FDMLP and FDMLB are provided in Table 2. The amount of protein linked to acid detergent fiber (ADF) was determined (AOAC, 2007) and used to estimate the chitin contents of DM, FDMLP and FDMLB (Finke, 2007). The chitin contents of DM, FDMLP and FDMLB were found as 4.20%, 2.74% and 2.81%, respectively.

Manure sampling and analyses: Manure samples were collected between the days 21-28, 28-35 and 35-42. Samples were taken 3 times a day from 3 cm below the surface of litter material. Feathers and other litter materials were carefully removed before taking the samples. For pH measurement, 10 g of fresh manure was extracted with 100 ml of distilled water for 2 h on a mechanical shaker and centrifuged at 3000 rpm for 10 min. The pH was immediately measured using a digital pH meter (HANNA HI8424 Portable pH/Mv meter) (Chung, 2017).

The dry matter and nitrogen contents of manure samples were measured in accordance with the methods specified in AOAC (2007).

Manure ammonia gas concentrations: On sampling days, a total of 300 g fresh manure samples obtained from each replicate were stored in 2.6 L sealed plastic containers in duplicate. Each box had a small hole in the middle of one side wall that was sealed by adhesive plaster. After being sealed in the containers, the samples were permitted to ferment for a period of 30 h at 32°C. After the fermentation period, manure samples were manually shaken for approximately 30 s to disrupt any crust formation on the surface of the slurry sample and to homogenize them. In the measurement, the adhesive plaster was punctured and 100 mL of the headspace air was sampled approximately 2.0 cm above the manure

samples at a rate of 100 mL/min. Level of ammonia gas of manures were measured by automatic gas measuring device (ProGasBadge BH-90A, China) (Park *et al.*, 2016; Lan *et al.*, 2017).

Table 2. Nutrient composition and concentrations of microorganisms in DM, FDMLP and FDMLB

Item	DM	FDMLP	FDMLB
Microorganisms' concentrations (log10 cfu/g)			
Total Mesophilic Aerobic Bacteria	Not detected	3.92	4.29
Lactobacillus	Not detected	2.99	2.45
Yeast-Mold	Not detected	Not detected	Not detected
Nutrient Composition			
Dry Matter, %	95.70	92.00	89.81
Crude Protein, %	76.20	49.28	49.06
Crude Fat, %	6.60	8.14	9.40
Crude Ash, %	7.30	7.81	7.83
Starch, %	3.30	3.73	1.44
Total Sugar, %	0.50	0.36	0.36
Metabolisable Energy, Kcal/kg (for poultry)	3515	2650	2660

Manure urease enzyme activity: The urease enzyme activity of manure samples was measured according to the methods of Karabulut and Canbolat (2005). For this purpose, 50 mg of ground dry manure sample was placed in a tube with a shaved mouth and 10 ml of buffered urea solution was added to it and shaken immediately by closing the lid. After waiting for 30 minutes in a 30ºC water bath, 10 ml of 0.1 N hydrochloric acid was added and cooled to room temperature rapidly and poured into a 100 ml beaker. The 100 ml beaker containing the sample was placed on the magnetic stirrer and the electrode of the pH meter was immersed into the solution in the beaker. In addition, 0.1% methyl orange indicator was dropped into the beaker and titrated with 0.1 N NaOH until the pH value was 4.7. The amount of 0.1 N NaOH consumed in the titration was recorded in ml. For the blind experiment, 10 ml of buffered urea solution and 10 ml of 0.1 N hydrochloric acid solution were placed in a glass-capped tube and 50 mg of fertilizer sample was added. After 30 min in ice water, it was titrated with 0.1 N NaOH solution in a 100 ml beaker. The 0.1 N hydrochloric acid consumed as a result of the titration was recorded in ml.

The urease enzyme activity was calculated using the following formula;

Urease activity (mg nitrogen/g/min at 30°C) = (1.4 x (a-b)) / (30 x SA)

a: Amount of 0.1 N NaOH solution spent in the main trial (ml)

b: Amount of 0.1 N NaOH solution spent in the blank trial (ml)

SA: Sample Amount (g)

Statistical analysis: The univariate general linear model using the SPSS statistical software (Version 17.0; Chicago, IL, United State) (SPSSWIN, 2007) was applied to the collected data with a model including stocking densities (SDs) and diets (Ds) and the interaction between SDs and Ds. Significant differences between treatment means were separated by Duncan's multiple range test (Duncan, 1955). All statements of significance were based on a P value of ≤ 0.05.

RESULTS AND DISCUSSION

Manure pH value, moisture and nitrogen contents: Effects of experimental treatments on broiler manure pH value are provided in Table 3.

Ds and interaction between SDs and Ds did not influence broiler manure pH value (Table 3). However, HSD significantly ($P \leq 0.001$) increased manure pH value as compared to that of broilers reared under NSD. This result is partially in agreement with the findings of Petek *et al.* (2014) who pointed out that HSD significantly increased litter pH value compared to that of broilers reared under NSD and low SD. In the present study, increasing manure pH value can be attributed to decreasing number of beneficial bacteria and increasing number of pathogenic bacteria (*E. coli, Clostridium spp., Staphylococcus aureus etc.*) as a result of stress caused by HSD, negatively affecting the microflora of the small intestine of broilers (Sugiharto, 2022). Increasing levels of *Clostridium spp.* species with urease enzyme activity in the small intestine increased the breakdown of urea into ammonia and thus may result in increased manure pH value (Endo *et al.*, 1999).

Effects of experimental treatments on the moisture content of broiler manure are given in Table 4.

Table 3. Effects of experimental treatments on broiler manure pH value

Stocking Densities (SDs), birds/m^2	Diets (Ds)	pH value		
		days 21-28	days 28-35	days 35-42
12	CONT	5.71	5.21	5.27
12	FDMLP	5.48	5.00	4.79
12	FDMLB	5.73	4.89	4.74
18	CONT	6.65	6.64	6.46
18	FDMLP	6.46	6.54	6.46
18	FDMLB	6.31	6.38	5.90
SEM		0.058	0.097	0.117
SDs				
12		5.64^b	5.04^b	4.93^b
18		6.47^a	6.52^a	6.27^a
SEM		0.051	0.080	0.129
Ds				
CONT		6.18	5.93	5.86
FDMLP		5.97	5.77	5.62
FDMLB		6.02	5.63	5.32
SEM		0.063	0.098	0.158
P values				
SDs		0.000	0.000	0.000
Ds		0.060	0.112	0.059
SDs x Ds Interaction		0.051	0.923	0.445

[a-b] Means indicated with different letters in the same column are significantly different ($P \leq 0.001$).
SEM: Standard Error of the Mean
[1] Data are means of fifteen replicates per treatment group

Table 4. Effects of experimental treatments on the moisture content of broiler manure, %

Stocking Densities (SDs), Birds/m^2	Diets (Ds)	Moisture Content		
		days 21-28	days 28-35	days 35-42
12	CONT	51.59	47.50	48.90
12	FDMLP	50.26	46.93	48.15
12	FDMLB	49.58	45.03	47.13
18	CONT	61.16	60.19	59.01
18	FDMLP	60.73	58.50	57.27
18	FDMLB	60.62	58.28	55.02
SEM		0.869	1.094	0.975
SDs				
12		50.48^b	46.49^b	48.06^b
18		60.84^a	58.99^a	57.10^a
SEM		0.802	1.064	1.109
Ds				
CONT		56.37	53.84	53.96
FDMLP		55.50	52.72	52.71
FDMLB		55.10	51.65	51.07
SEM		0.982	1.303	1.358
P values				
SDs		0.000	0.000	0.000
Ds		0.646	0.497	0.329
SDs x Ds Interaction		0.869	0.899	0.846

[a-b] Means indicated with different letters in the same column are significantly different ($P \leq 0.001$).
SEM: Standard Error of the Mean
[1] Data are means of fifteen replicates per treatment group

As can be inferred from Table 4, the moisture content of broiler manure was not affected by Ds and interaction between SDs and Ds. However, HSD significantly ($P \leq 0.001$) enhanced manure moisture content as compared to that of broilers reared under NSD. This finding is partially consistent with the results of Petek *et al.* (2014), Astaneh *et al.* (2018) and Kang *et al.* (2018) indicating that increasing stocking density significantly enhanced litter moisture content of broilers compared to that of broilers reared under NSD. HSD increases body temperature of broilers (Petek *et al.*, 2014). Broilers try to stabilize their body temperatures with respiratory tracts. However, they drink more water because they are forced to reduce the heat stress caused by HSD by respiration. Excess water consumption causes to expel excess water with manure of broilers and thus increase the moisture content of manure (Orakpoghenor *et al.*, 2021).

Effects of experimental treatments on the nitrogen content of broiler manure are summarized in Table 5.

Table 5. Effects of experimental treatments on the nitrogen content of broiler manure, %

Stocking Densities (SDs), bird/m^2	Diets (Ds)	Nitrogen Content		
		days 21-28	days 28-35	days 35-42
12	CONT	2.68	2.73	4.39
12	FDMLP	2.66	2.56	4.80
12	FDMLB	2.55	2.43	4.67
18	CONT	2.48	2.42	4.75
18	FDMLP	2.79	2.51	4.81
18	FDMLB	2.49	2.55	4.65
SEM		0.060	0.038	0.044
SDs				
12		2.63	2.57	4.62
18		2.59	2.50	4.74
SEM		0.088	0.50	0.056
Ds				
CONT		2.58	2.58	4.57
FDMLP		2.72	2.54	4.81
FDMLB		2.62	2.49	4.66
SEM		0.106	0.062	0.068
P values				
SDs		0.759	0.323	0.151
Ds		0.404	0.682	0.067
SDs x Ds Interaction		0.533	0.098	0.119

SEM: Standard Error of the Mean

[1] Data are means of fifteen replicates per treatment group

SDs, Ds and interaction between SDs and Ds did not influence the nitrogen content of broiler manure (Table 5). This finding is in agreement with the result of Naseem *et al.* (2021) that showed that the supplementation of probiotics to laying hen diets did not affect the nitrogen content of their manures. In the present study, similar nitrogen excretions with manure were attributed to the similar crude protein contents of the diets used in the experiment. Therefore, the same level of crude protein may have consumed and thus the same amount of nitrogen may be excreted.

Manure ammonia gas concentration: Effects of experimental treatments on ammonia gas concentration of broiler manure are provided in Table 6.

HSD significantly increased the ammonia gas concentrations of broiler manures between the days 21-28 ($P \leq 0.001$), 28-35 ($P \leq 0.05$) and 35-42 ($P \leq 0.001$) as compared to those of broilers reared under NSD (Table 6). HSD causes stress by increasing body temperature of broilers, restricting their range of motion and deteriorating indoor air quality. Increasing stocking density has various adverse effects on the welfare and the small intestinal microflora of broilers that results in the overgrowth of pathogenic bacteria and depresses the growth of beneficial bacteria, which shifts the bacteria of the small intestine to a state of dysbiosis (Kridtayopas *et al.*, 2019).

As a result of this, number of beneficial bacteria (Lactic Acid Bacteria, LAB) decreases and number of pathogenic bacteria (*E. coli, Clostrodium perfringes, Staphylococcus aureus etc.*) increases. Increasing ammonia gas concentration at HSD could be attributed to increased number of pathogenic bacteria such as

Clostridium with urease enzyme activity in the small intestine microflora of broilers as a result of the deteriorated small intestine microflora at HSD (Endo *et al.*, 1999).

Table 6. Effects of experimental treatments on the ammonia gas concentration of broiler manure, ppm

Stocking Densities (SDs), birds/m^2	Diets (Ds)	Ammonia Gas Concentration		
		days 21-28	days 28-35	days 35-42
12	CONT	7.00	30.20	45.50
12	FDMLP	6.50	25.60	36.00
12	FDMLB	5.67	28.20	39.00
18	CONT	9.67	34.80	56.50
18	FDMLP	8.50	28.40	45.50
18	FDMLB	7.80	29.60	48.00
SEM		0.352	1.157	1.509
SDs				
12		6.39^b	28.00^b	40.17^b
18		8.66^a	30.93^a	50.00^a
SEM		0.337	1.609	1.382
Ds				
CONT		8.33^a	32.50^a	51.00^a
FDMLP		7.50^b	27.00^b	40.75^b
FDMLB		6.73^c	28.90^b	43.50^b
SEM		0.431	1.971	1.693
P values				
SDs		0.000	0.021	0.000
Ds		0.047	0.016	0.001
SDs x Ds Interaction		0.850	0.848	0.910

$^{a-c}$ Means indicated with different letters in the same column are significantly different ($P \leq 0.05$; $P \leq 0.01$; $P \leq 0.001$).
SEM: Standard Error of the Mean
[1] Data are means of fifteen replicates per treatment group

Feeding with the FDMLP and FDMLB diets significantly reduced broiler manure ammonia gas concentrations between the days 21-28 ($P \leq 0.05$), 28-35 ($P \leq 0.05$) and 35-42 ($P \leq 0.01$) as compared to those of broilers fed with CONT diet (Table 6). Although there is no previous study related to the effects of the dietary supplementation of fermented insects to broiler diets on manure ammonia gas concentration, decreasing manure ammonia gas concentration may be related to the combined effects of probiotics, chitooligosaccharides as prebiotic and short chain fatty acids as degradation products of chitin (Kwon *et al.*, 2020) and AMPs (Benzertiha *et al.*, 2020) in the FDMLP and FDMLB diets, which have the beneficial functions on microbial balance in the small intestine of broilers by enhancing beneficial bacteria such as *Lactobacillus* spp. and inhibiting pathogenic bacteria such as *Clostridium* with urease enzyme activity in the small intestine of broilers (Kridtayopas *et al.*, 2019; Benzertiha *et al.*, 2020). Further, FDMLP and FDMLB, that have synbotic properties, in diets may have a potential in reducing ammonia emissions. A lower ammonia excretion in manure can result in better litter and air quality that allow a better animal welfare and the positively environmental impacts such as greenhouse gas emissions (Leone *et al.*, 2023)

No significant interaction was observed between SDs and Ds in terms of ammonia gas concentration of broiler manure between the days 21-28, 28-35 and 35-42 (Table 6).

Manure urease enzyme activity: Effects of experimental treatments on broiler manure urease enzyme activity are provided Table 7.

As can be inferred from Table 5, HSD significantly increased broiler manure urease enzyme activity between the days 21-28 ($P \leq 0.001$), 28-35 ($P \leq 0.001$) and 35-42 ($P \leq 0.001$) (Table 7). HSD-induced stress factors (increased temperature, humidity and corticosteroid stress hormone levels) increased the number of pathogenic bacteria such as *Clostridium* with urease enzyme activity in the small intestine of broilers, thus, increased the urease enzyme activity of their manure (Sugiharto, 2022). Manure urease enzyme activity was significantly decreased by the FDMLP and FDMLB diets between the days 21-28 ($P \leq 0.001$), 28-35 ($P \leq 0.001$) and 35-42 ($P \leq 0.001$) as compared to CONT diet (Table 7). Although there is no previous research related to the effects of the dietary supplementation of

fermented insects to broiler diets on manure urease enzyme activity, decreasing manure urease enzyme activity can partially be explained by the combined effects of probiotics (Cengiz *et al.*, 2015), chitooligosaccharides as prebiotic and short chain fatty acids as degradation products of chitin (Kwon *et al.*, 2020) and AMPs (Benzertiha *et al.*, 2020) in the FDMLP and FDMLB diets, which inhibit the growth and development of pathogenic bacteria such as *Clostridium* with urease enzyme activity in the small intestine of broilers (Kridtayopas *et al.*, 2019; Benzertiha *et al.*, 2020) and thus reduced the urease enzyme activity of manure (Sugiharto, 2022). There is a significant effect of the interaction between SDs and Ds on broiler manure urease enzyme activity between the days 21-28 ($P \leq 0.001$), 28-35 ($P \leq 0.001$) and 35-42 ($P \leq 0.01$) (Table 7). The FDMLP and FDMLB diets significantly reduced the urease enzyme activity of manure of broilers reared under NSD and HSD during the above-mentioned periods.

Table 7. Effects of experimental treatments on broiler manure urease enzyme activity, (mg nitrogen/g/min at 30°C)

Stocking Densities (SDs), birds/m^2	Diets (Ds)	Urease Enzyme Activity		
		days 21-28	days 28-35	days 35-42
12	CONT	-0.178^{a}	0.242^{a}	0.760^{a}
12	FDMLP	-0.796^{c}	-0.776^{c}	0.370^{c}
12	FDMLB	-0.278^{b}	-0.270^{b}	0.394^{b}
18	CONT	0.128^{a}	0.368^{a}	0.966^{a}
18	FDMLP	0.062^{c}	0.246^{c}	0.840^{c}
18	FDMLB	0.082^{b}	0.310^{b}	0.906^{b}
SEM		0.044	0.057	0.037
SDs				
12		-0.417^{b}	-0.268^{b}	0.508^{b}
18		0.091^{a}	0.308^{a}	0.904^{a}
SEM		0.017	0.020	0.021
Ds				
CONT		-0.025^{a}	0.305^{a}	0.863^{a}
FDMLP		-0.367^{c}	-0.265^{c}	0.605^{c}
FDMLB		-0.098^{b}	-0.020^{b}	0.650^{b}
SEM		0.021	0.030	0.031
P values				
SDs		0.000	0.000	0.000
Ds		0.000	0.000	0.000
SDs x Ds Interaction		0.000	0.000	0.003

$^{a-c}$ Means indicated with different letters in the same column are significantly different ($P \leq 0.01$; $P \leq 0.001$).
$^{a-c}$ Small letters on the right show the interaction between SDs and DTs
SEM: Standard Error of the Mean, [1] Data are means of fifteen replicates per treatment group

Conclusion: In conclusion, FDMLP and FDMLB can be utilized as new antibacterial feed additives in broiler diets in terms of reducing of ammonia gas concentration and urease enzyme activity of broiler manure regardless of stocking density. Considering the increasing interest of people in more environmentally friendly products and in animal health and welfare, more studies need to highlight the mode of action of FDMLP and FMBLB on manure quality of broilers under various environmental circumstances. Moreover, there is a need for further studies to ascertain the effects of dietary supplementation of FDMLP and FDMLB in combination as an antibacterial feed additive in broilers reared under HSD on manure quality and ammonia gas emission that is a global problem.

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REFERENCES

Adegbeye, M.J., M.M.M.Y. Elghandour, J.C. Monroy, T.O. Abegunde, A.Z.M. Salem, A. Barbabosa-Pliego and T.O. Faniyi (2019). Potential influence of yucca extract as feed additive on greenhouse gases emission for a cleaner livestock and aquaculture farming - A review. J. Clean. Prod. 239(1):1-11. DOI: https://doi.org/10.1016/j.jclepro.2019.118074

Al-Kerwi, M.S.M., O. Mardenli, M.S.M. Jasim and M.A. Al-Majeed (2022). Effects of harmful gases emitted from poultry houses on productive and health performance. IOP Conference Series: Earth Environ. Sci. 1060 012082. DOI: https://doi.org/10.1088/1755-1315/1060/1/012082

Anderson, K., P.A. Moore, J. Martin, and A.J. Ashworth (2020). Effect of a new manure amendment on ammonia emissions from poultry litter. Atmosphere. 11(3):1-14. DOI: https://doi.org/10.3390/atmos11030257

AOAC (2007). Association of Official Analytical Chemists. 18 th. Edition, W.D.C. USA.

Astaneh, I.Y., M. Chamani, S.N. Mousavi, A.A. Sadeghi and M.A. Afshar (2018). Effects of stocking density on performance and immunity in Ross 308 broiler chickens. Kafkas Üni. Vet. Fak. Derg. 24(4):483-489. DOI: https://doi.org/10.9775/kvfd.2017.18869

Ayiti, O.E. and O.O. Bablola (2022). Factors influencing soil nitrification process and the effect on environment and health. Front. in Sustain. Food Syst. 6:821994. DOI: https://doi.org/10.3389/fsufs.2022.821994.

Benzertiha, A., B. Kierończyk, P. Kolodziejski, E. Pruszyńska-Oszmalek, M. Rawski and Józefiak, A. (2020). *Tenebrio molitor* and *Zophobas morio* full-fat meals as functional feed additives affect broiler chickens' growth performance and immune system traits. Poult. Sci. 99(1):196-206. DOI: https://doi.org/10.3382/ps/pez450

Boroojerdi, N.N. and M. Rajabzadeh (2021). The effect of replacing dried mealworm with soybean meal on growth performance and carcass characteristics of broiler chickens. Res. Square. 1-16. DOI: https://doi.org/10.21203/rs.3.rs-782326/v1

Chung, T.H. (2017). pH, ammonia flux, and total nitrogen in duck litter with chemical blends - a field study. Brazil. J. Poult. Sci. 19(4):733-736. DOI: https://doi.org/10.1590/1806-9061-2017-0491

Cengiz, O., B.H. Koksal, O. Tatli, O. Sevim and U. Ahsan (2015). Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora and stress indicators of broilers. Poult. Sci. 94(10):2395-2403.DOI: https://doi.org/10.3382/ps/pev194

Cook, K.L., M.J. Rothrock Jr, M.A. Eiteman, N. Lovanh and K. Sistani (2011). Evaluation of nitrogen retention and microbial populations in poultry litter treated with chemical, biological or adsorbent amendments. J. Environ. Man. 92(7):1760-1766. DOI:https://doi.org/10.1016/j.jenvman/2011.02.005

Duncan, D.B. (1955). Multiple range test and multiple F tests. Biometrics. 11(1):1-42

Eglite, S., A. Ilgaza and M. Butka (2021). Reduction of ammonia emissions by applying probiotics on litter in a commercial breeding poultry house. Agron. Res. 19 (S2):1015-1022. DOI: https://doi.org/10.15159/AR.21.069

Elahi, U., C.C. Xu, J. Wang, J. Lin, S.G. Wu, H.J. Zhang and G.H. Qi (2022). Insect meal as a feed ingredient for poultry. Anim. Biosci. 35(2):332-346. DOI: https://doi.org/10.5713/ab.21.0435

Endo, T., M. Nakano, S. Shimizu, M. Fukushima and S. Miyoshi (1999). Effects of a Probiotic on the lipid metabolism of cocks fed on a cholesterol-enriched diet. Biosci. Biotechn. Biochem. 63(9):1569-1575. DOI: https://doi.org/10.1271/bbb.63.1569

Finke, M. (2007). An estimate of chitin in raw whole insects. Zoo Biol. 26(2):105-115. DOI: https://doi.org/10.1002/zoo.20123

Hong, J., T. Han and Y.Y. Kim (2020). Mealworm (*Tenebrio molitor* larvae) as an alternative protein source for monogastric animal: a Review. Anim. 10(2068):1-20. DOI: https://doi.org/10.3390/ani10112068

Islam, M.M. and C.J. Yang (2017). Efficacy of mealworm and super mealworm larvae probiotics as an alternative to antibiotics challenged orally with *Salmonella* and *E. coli* infection in broiler chicks. Poult. Sci. 96 (1):27-34. DOI: https://doi.org/10.3382/ps/pew220

Jiang, J., D.S. Stevenson, A. Uwizeye, G. Tempio and M.A. Sutton (2021). A climate-dependent global model of ammonia emissions from chicken farming. Biogenosci. 18:135-158. DOI: https://doi.org/10.5194/bg-18-135-2021

Kang, H.K., S.B. Park, J.J. Jeon, H.S. Kim, S.H. Kim, E. Hong and C. H. Kim (2018). Effect of stocking

density on laying performance, egg quality and blood parameters of Hy-Line Brown laying hens in an aviary system. Europ. Poult. Sci. 82:1-13. DOI: https://doi.org/10.1399/eps.2018.245

Karabulut, A. and Ö. Canbolat (2005). Determination of urease activity. Feed evaluation and analysis methods. Uludag Uni. Pub. 88-91 (Original in Turkish)

Khalil, F., R.R. Ibrahim, H.H. Emeash and A.H.A. Hassan (2021). Probiotic supplementation alleviated stress and improved performance, meat quality sensory acceptability and microbiological status of broilers. J. Adv. Vet. Res. 11(2):93-101

Kridtayopas, C., C. Rakangtong, C. Bunchasak and W. Loongyai (2019). Effect of prebiotic and synbiotic supplementation in diet on growth performance, small intestinal morphology, stress, and bacterial population under high stocking density condition of broiler chickens. Poult. Sci. 98(10):4595-4605. DOI: https://doi.org/10.3382/ps/pez152

Kwon, G.T., H.G. Yuk, S.J. Lee, Y.H. Chung, H.S. Jang, J.S. Yoo, K.H. Cho, H. Kong and D. Shin (2020). Mealworm larvae (*Tenebrio molitor* L.) exuviae as a novel prebiotic material for BALB/c mouse gut microbiota. Food Sci. Biotechnol. 29(4):531–537. DOI: https://doi.org/10.1007/s10068-019-00699-1

Lan, R.X., S.I. Lee and I.H. Kim (2017). Effects of *Enterococcus faecium* SLB 120 on growth performance, blood parameters, relative organ weight, breast muscle meat quality, excreat microbiota shedding, and noxiousgas emission in broilers. Poult. Sci. 96:3246-3253. DOI: https://doi.org/10.3382/ps/pex101

Lee, J.H., T.K. Kim, Ji Y. Cha, H.W. Yang, H.I. Yong and Y.S. Choi (2022). How to develop strategies to use insects as animal feed: digestibility, functionality, safety and regulation. J. Anim. Sci. Technol. 64(3):409-431. DOI: https://doi.org/10.5187/jast.2022.e27

Leone, F. and V. Ferrante (2023). Effects of prebiotics and precision biotics on performance, animal welfare and environmental impact. A review. Sci. Total Environ. 901: 165951. DOI: https://doi.org/10.1016/j.scitotenv.2023.165951

Liu, X., G. Cao, K. Qiu, Y. Dong and C. Hu (2023). *Lactobacillus plantarum* decreased ammonia emissions through modulating cecal microbiotain broilers challenged with ammonia. Anim. 13(7):2739. DOI: https://doi.org/10.3390/ani13172739

Luparelli, A.V., J. Hadj Saadoun, V. Lolli, C. Lazzi, S. Sforza and A. Caligiani (2022). Dynamic changes in molecular composition of black soldier fly prepupae and derived biomasses with microbial fermentation. Food Chem. X14. 1-9. DOI:https://doi.org/10.1016/j.fochx.2022.100327

Mahardhika, B.P., R. Mutia and M. Ridla (2019). Efforts to reduce ammonia gas in broiler chicken litter with the use of probiotics. IOP Conf. Series: Earth Environ. Sci. 399:1-7. DOI: https://doi.org/10.1088/1755-1315/399/1/012012

Menezes, A.G.T., C.L. Ramos and D.R. Dias (2018). Combination of probiotic yeast and lactic acid bacteria as starter culture to produce maize-based beverages. Food Res. Int. 111:187-197. DOI: https://doi.org/10.1016/j.foodres.2018.04.065

Mulyono, M., V.D. Yunianto, N. Suthama and D. Sunarti (2019). The effect of fermentation time and *Trichoderma* levels on digestibility and chemical components of Black Soldier fly (*Hermetia illucens*) larvae. Livestock Res. Rural Develop. 31(10):1-4

Naseem, S. and A. King (2018). Ammonia production in poultry houses can affect of humans, birds, and the environment – techniques for its reduction during poultry production. Environ. Sci. Pollution Res. 25:15269-15293. DOI: https://doi.org/10.1007/s11356-018-2018-y

Naseem, S., N. Willits and A.J. King (2021). Varying combinations of *Lactobacillus* species: impact on laying hens' performance, nitrogenous compounds in manure, serum profile, and uric acid in the liver. Translational Anim. Sci. 5(2):1-14. DOI: https://doi.org/10.1093/tas/txab018

Orakpoghenor, O., N.E. Ogbuagu and L. Sa'Idu (2021). Effect of environmental temperature on water intake in poultry. In Adv. in Poult. Nutr. Res. IntechOpen. 87-93. DOI: https://doi.org/10.5772/intechopen.95695

Park, J.W., J.S. Jeong, S.I. Lee and I.H. Kim (2016). Effect of dietary supplementation with a probiotic (*Enterococcus faecium*) on production performance, excreta microflora, ammonia emission, and nutrient utilization in ISA brown laying hens. Poult. Sci. 95(12):2829-2835. DOI: https://doi.org/10.3382/ps/pew241

Petek, M., H. Üstüner and D. Yeşilbağ (2014). Effects of stocking density and litter type on litter quality and growth performance of broiler chicken. Kafkas Üni. Vet. Fak. Derg. 20(5): 743-748. DOI: https://doi.org/10.9775/kvfd.2014.11016

Rehman, A., M. Arif, N. Sajjad, M.Q. Al-Ghadi, M. Alagawany, M.E. Abd El-Hack, A.R. Alhimaidi, S.S. Elnesr, B.O. Almutairi, R.A. Amran, E.O.S. Hussein and A.A. Swelum (2020). Dietary effect of probiotics and prebiotics on broiler performance, carcass and immunity. Poult. Sci.

99:6946-6953. DOI: https://doi.org/10.1016/j.psj.2020.09.043

Ross 308 AP. (2017). Performance objectives 2017. https://la.avaigen.com/assets/Tech-Center/Ross-Broiler/Ross308AP-Broiler-PO-2017-EN-pdf

Senol, M., H. Nadaroglu, N. Dikbas and R. Kotan (2014). Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*. Annals Clin. Microbiol. Antimic. 13(35):1-7. DOI: http://doi.org/10.1186/s12941-014-0035-3

Shi, H., M. Zhang, W. Wang and S. Devahastin (2020). Solid-state fermentation with probiotics and mixed yeast on properties of okara. Food Biosci. 36:1-8. DOI: https://doi.org/10.1016/j.fbio.2020.100610

Son, Y.J., I.K. Hwang, C.W. Nho, S.M. Kim and S.H. Kim (2021). Determination of carbohydrate composition in mealworm (*Tenebrio molitor* L.) larvae and characterization of mealworm chitin and chitosan. Foods. 10(640):1-15. DOI: https://doi.org/10.3390/foods10030640

SPSSWIN. (2007). SPSS for Windows 6.1.4. SPSSWIN. Istanbul, Turkey

Such, N., G. Csitári, P. Stankovics, L. Wágner, I.A. Koltay, V. Farkas, L. Pál, P. Strifler and K. Dublecz (2021). Effects of probiotics and wheat bran supplementation of broiler diets on the ammonia emission from excreta. Anim. 11(9):2703. DOI: https://doi.org/10.3390/ani11092703

Sugiharto, S. (2022). Dietary strategies to alleviate high-stocking-density-induced stress in broiler chickens-a comprehensive review. Arch. Anim. Breeding. 65(1):21-36. DOI: https://doi.org/10.5194/aab-65-21

Wang, Q., Y. Miao and L. Wang (2020). Regional transport increases ammonia concentration in Beijing, China. Atmosphere. 11(6):563. DOI: https://doi.org/10.3390/atmos11060563

Zou, Q., X. Fan, Y. Xu, T. Wang and D. Li (2022). Effects of dietary supplementation probiotic complex on growth performance, blood parameters, fecal harmful gas and fecal microbiota in AA+male broilers. Front. in Microbiol. 13:1088179. DOI: https://doi.org/10.3389/fmicb.2022.1088179.