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Effect of dietary housefly maggot extract on egg production and egg quality in laying hens under a digital livestock system

*Victor A. Zammit[1], Sang O. Park[2]**

[1]*Metabolic Biochemistry, Warwick Medical School, University of Warwick, Coventry, UK*
[2]*Institute of Animal Life Science, Kangwon National University, Chuncheon-si, Gangwon State, Republic of Korea*

**Corresponding author: bspark@kangwon.ac.kr*

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Abstract: Antibiotics in poultry feed are banned in many countries owing to their side effects, and insects containing antimicrobial peptides are proven to have potential as antibiotic alternatives in such feed. Thus, the primary objective of this study was to investigate the effects of dietary housefly maggot extract (HME) as the biofunctional material for antibiotic replacement in laying hens. The biofunctional properties of HME on egg production, egg quality, blood biomarkers of immune function, and microbiota were evaluated over a period of 40 to 50 weeks in laying hens under a digital livestock system. A total of 120 forty-week-old Hyline Brown laying hens were randomly divided into four groups with thirty replicates as follows: no added HME (CON), positive control (PC, 8 ppm of avilamycin), 100 ppm of HME (HME100), and 150 ppm of HME (HME150). Egg production and egg weight increased significantly in the HME150, HME100, PC, and CON groups ($P < 0.05$), but there were no differences between the HME and PC groups. Egg quality, such as eggshell thickness, eggshell strength, Haugh unit, and albumin height, were significantly improved in the HME and PC groups than in the CON group ($P < 0.05$). A blood biomarker of immune function, IgG, was significantly higher in the HME and PC groups than the CON group ($P < 0.05$), but there were no differences between the HME and PC groups. Blood corticosterone and heterophil to lymphocyte ratio were significantly lower in the HME and PC groups than in the CON group ($P < 0.05$). Microbiota *Lactobacillus* in the faeces were significantly higher in the HME and PC groups than in the CON group ($P < 0.05$). The faecal total aerobic bacteria, *Escherichia coli*, and coliform counts were significantly lower in the HME and PC groups than in the CON group ($P < 0.05$). Consequently, the HME showed the same significant effects as antibiotics on improving egg production and egg quality in laying hens. These results show that 100 ppm of HME can be used as a biofunctional material for an effective alternative to antibiotics in laying hens under a digital livestock system to improve egg production and quality by stimulating their immune functions and balancing the microbiota populations.

Keywords: poultry; insect; antimicrobial peptide; blood biomarker; microbiota

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Climate change and population growth are two of the major factors that threaten the sustainability of livestock production as animal food. It has been predicted that by 2050, the global population will increase to 9.9 billion and food production will increase by 70%. As a result of climate change and a ban on using antibiotics in livestock feed, there may be increased incidents of animal stress, which may decrease their immune functions and food production (Park 2022).

Given the extensive concerns regarding environmental and food safety, the use of antibiotics in laying hens has been banned in many countries, and numerous studies have been conducted in recent years to investigate the potential of other additives as replacements for antibiotics; of these, insect-derived products have garnered much attention owing to their environmentally friendly properties and numerous biological benefits (Qui 2023). Insects constitute about three-quarters of all organisms on Earth and are an important natural feed source for poultry; hence, they are emerging as novel antimicrobial peptide immunomodulators to replace antibiotics in feedstuffs (Wu et al. 2018). Insect members from orders such as the Diptera (housefly, black soldier fly) contain antimicrobial peptides as natural immunomodulators and have excellent nutritional value, which can greatly lower the cost of poultry production (Lokaewmanee et al. 2023). Natural immunomodulators activate the host defense system in a damaged body and suppress nonspecific immune responses, such as autoimmune disorders (Cheng et al. 2014). In the Republic of Korea, about 60 million tons of livestock manure is generated every year, causing environmental pollution due to livestock odours. The HooinEcobio Co. Ltd. (Hongseong, Republic of Korea) developed and commercialized a biological technology to treat livestock manure using housefly maggots and black soldier flies (Park 2023). The housefly maggot (*Musca domestica*) is considered a natural feed source for poultry owing to its high content of over 50% proteins, fats, energy, vitamins, and minerals (Hwangbo et al. 2009). It has been reported that fresh or dried housefly maggots can replace fishmeal and soybean meal without affecting the growth performances, egg quality, and egg production of poultry (Teguia et al. 2002). Insects such as the housefly and black soldier fly are known to contain important antimicrobial peptides with innate immunity, such as defensins, cecropins, drosocins, attacins, and ponericins (Wu et al. 2018; Moretta et al. 2020). Housefly maggot extract (HME) is known as a biofunctional material, which contains a potent 5–22 kDa antimicrobial peptide against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *enterococci* strains (Park et al. 2010; Park 2023). However, there is only one report on the improvement of growth performance and immune functions by dietary HME as a biofunctional material for antibiotic replacement in diets in the poultry industry. It has been reported that dietary HME can replace antibiotics in broiler chickens and improve their growth performances as an immunomodulator. The fact that HME, along with a digital livestock system, has an antibiotic-substituting effect as an immunomodulator means that livestock production can be enhanced without using antibiotics and that antibiotic-free, safe animal food production is possible (Cheng et al. 2014; Park 2023; Park and Zammit 2023)

Regarding the immune functions and microbiota of animals, the health of laying hens is a prerequisite for animal welfare, high productivity, and sustainable egg production. This affects the egg production and egg quality of laying hens and has been well-described for the development of immune cells in the blood, lymph, and other tissues in many vertebrates, including birds (Alkie et al. 2019). However, the biological effects of HME as biofunctional materials for improving blood biomarkers of immune function and microbiota related to egg production and egg quality have rarely been reported. Therefore, the present study evaluates the biofunctional properties of HME containing antimicrobial peptides for improving egg production and egg quality in laying hens under a digital livestock system.

MATERIAL AND METHODS

Dietary HME supplements

The manufacture of housefly maggot extract (HME) was conducted according to the method reported by Park (2023). The extraction is the process by which lipids are completely removed from the housefly maggot and get defatted housefly maggot. Briefly, housefly maggots provided by HooinEcobio Co., Ltd (Hongseong, Chungcheongnam-do, Korea) were compressed

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at 1 000 psi, and then lipid was completely removed using hexane to obtain defatted housefly maggot. HME, including 5 kDa antibacterial peptide was obtained by a reflux condensing system (Eyela N-1000, Rikakikai Co., Tokyo, Japan) after mixing defatted housefly maggot with absolute ethanol. The dietary supplements (HME 1 000 ppm; 1 000 mg/zeolite kg) were prepared by spraying HME dissolved in ethanol evenly on zeolite, mixing well, and then air drying. HME is a non-nutritive additive with zero or very few calories and does not provide proximate nutrients, amino acids, and minerals. This is because HME was made by dissolving HME in ethanol and spray-drying it into zeolite as a carrier that does not contain any nutrients.

Experimental design and animal feeding

Animal experiments were conducted in compliance with the scientific and ethical regulations presented by the EC Directive 1986, 86/609/EEC, and approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University, Republic of Korea (KNU 015017). In animal experiments, age was an important factor affecting the performance and egg quality. According to the Hy-Line management guide, in March 2023, 40-week-old Hy-Line Brown laying hens at an appropriate age were used in this experiment for periods of 10 weeks to more than 40 to 50 weeks. A total of 120, 40-week-old Hyline Brown laying hens were randomly allocated among four treatment groups: no added HME (CON), positive control (PC, 8 ppm of avilamycin), 100 ppm of HME (HME100), and 150 ppm of HME (HME150). In this study, the antibiotic avilamycin (Avilamix; Ctcbio Co., Ltd South Korea) was used as a substance challenge agent for measuring the antimicrobial effects and blood biomarkers of immune function. The amount of added HME was determined on the basis of research results of Park (2023). The diet's portion size with added HME was controlled using yellow corn grain. The experimental animals were housed in wire cages (size: 40 × 40 × 40 cm), with 30 replicates per group, and reared individually in separate cages under a digital livestock system (Park and Zammit 2023).

The formulas and chemical compositions of the experimental diets are shown in Table 1. These diets were formulated to meet the daily nutrient requirements of the laying hens according to the recommendations of the NRC (1994) so as to contain equal amounts of protein (17.10%) and apparent metabolizable energy (AME: 11.80 KJ). The chemical compositions and AME of the diets were analyzed based on the previously presented methods (Park et al. 2017). The feed and drinking water were provided to animals *ad libitum* for 10 weeks (from the 40th to 50th week of age). The poultry housing temperature, relative humidity, and lighting availability were maintained at 22–25 °C, 60–65%, and 20 lux light intensity via a red light-emitting diode (LED) for 16 h light/8 h darkness, respectively, during the entire experimental period. The laying hens were also subjected to routine management activities, such as vaccination and medication.

Table 1. Ingredients and chemical composition of the experimental diet for laying hens (as-is fed basis)

Ingredients	%
Yellow corn grain	53.4
Soybean oil meal (44% protein)	20.4
Corn gluten meal	4.13
Wheat grain	10.0
Soybean oil	1.00
Limestone	9.87
Dicalcium phosphate	0.50
Choline chloride	0.30
DL-Methionine (50%)	0.20
L-Lysine hydrochloride (78%)	0.05
Mineral-vitamin premix[1]	0.15
Analysed chemical composition	
Apparent metabolic energy (KJ)	11.80
Crude protein	17.1
Lysine	0.78
Methionine	0.56
Methionine-cysteine	0.75
Calcium	3.80
Available phosphorous	0.37

[1]Provided per kg of diet: vitamin A (retinyl acetate), 10 500 IU; vitamin D_3 (cholecalciferol), 4 100 IU; vitamin E (DL-α-tocopheryl acetate), 45 mg; menadione, 3.0 mg; thiamin, 2.5 mg; riboflavin, 5.0 mg; pyridoxine, 4.0 mg; cyanocobalamin, 0.02 mg; niacin, 44 mg; pantothenic acid, 17 mg; folic acid, 1.5 mg; biotin, 0.18 mg; Fe (ferrous sulfate), 80 mg; zinc (zinc oxide), 80 mg; Mn (manganese sulfate), 70 mg; Cu (copper sulfate), 7 mg; I (calcium iodate), 1.20 mg; Se (sodium selenite), 0.30 mg; Co (cobalt), 0.70 mg

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Egg production and egg quality

Fresh feeds were provided to the animals every day. The egg production and egg weights were recorded daily, the feed intake was recorded weekly per replicate, and the daily feed intake was presented as values of the total weekly feed intake divided by seven days. The egg quality was investigated by randomly collecting 15 egg samples from each treatment group on the same day every week for 40 to 50 weeks and expressed as the average value. The eggshell thickness was measured at three points on the surface (large end, middle, and small end of the egg) using a dial pipe gauge (Model 7360, Mitutoyo Co., Kawasaki 213, Japan) and expressed as the average value. The eggshell strength was determined using a texture analyzer (Model T2100C, Food Technology Corp., Rockville, MD, USA). The Haugh unit (HU) was calculated using the equation shown below after measuring the egg albumin height (ORKA Food Technology Ltd., Ramat HaSharon, Israel) and egg weight using a HU tester (FHL Co., Japan).

$$HU = 100 \log (H + 7.57 - 1.7\ W^{0.37}) \quad (1)$$

where:
HU – Haugh unit;
H – albumin height (mm);
W – egg weight (g).

The yolk colour was measured in the grade range of 1 to 15 points using Roche's yolk colour fan (Hoffman-La Roche Ltd., Basel, Switzerland).

Blood biomarkers

At 45, 47, and 50 weeks of age, 10 laying hens were randomly selected from each group to measure the blood biomarkers of the immune function. Blood was drawn using a general syringe without blood coagulation factor through cardiac puncture. The blood serum was then separated by centrifugation at 25 000 *g* for 15 minutes. The serum samples were stored at –90 °C until biochemical analyses. The immunoglobulin (IgG) concentration was measured using a chicken ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). After treating the blood samples according to manufacturer protocols, their absorbance values were determined at 450 nm using a precision microplate reader (Molecular Devices Inc., New York, NY, USA) to calculate the quantity of antibodies. Corticosterone concentrations were next measured using the HS EIA kit (Enzyme immunoassay kit, IDS, Boldon, UK) according to manufacturer protocol, biochemically pretreated, and the absorbance values were measured at 450 nm using a precision microplate reader (Molecular Devices Inc., New York, NY, USA) for quantification. Leukocytes, white blood cells, heterophils, and lymphocytes were determined using a haematological analyzer (HEMAVET® HV950FS, Drew Scientific Inc., USA). The heterophils (H) and lymphocytes (L) were isolated via a light microscope, and 100 leukocytes per slide and 200 cells per hen were counted; then, the H : L ratios were calculated (Cheng et al. 2001).

Microbiota

The samples of fresh faeces were collected from 10 laying hens selected randomly from each group after the conclusion of the experimental period to measure the microbiota counts in faeces. Fresh faecal samples were mixed with fertilized phosphorus-buffered saline (0.1M, pH 7.0) solution and diluted 10 times (1 : 9, w/v) to obtain concentrations in the range of 10^{-2} to 10^{-7}. The samples were plated onto selective media plates for *Lactobacillus* (MRS agar, Oxoid, Basingstoke, UK), *Escherichia coli* (McConkey purple agar, Difco Laboratories Inc, Franklin Lakes, NJ, USA), coliform bacteria (Violet red bile agar, Difco Laboratories Inc, Franklin Lakes, NJ, USA), and total aerobic bacteria (Nutrient agar, Difco). Aerobic cultures of the *E. coli*, coliform bacteria, and total aerobic bacteria were maintained at 37 °C for 24 h. *Lactobacillus* was cultured under anaerobic conditions using anaerobic jars sealed with Anaero Gen sachets at 37 °C for 48 h. The number of faecal microbiota colonies was described in terms of the bacterial counts per gram of faeces (colony-forming unit, cfu/g, fresh faeces).

Statistical analyses

Statistical analyses of the results were performed using IBM SPSS Statistics v17.0 (SPSS Inc., Chicago, IL, USA), one-way ANOVA, and Duncan's multiple range tests. Since there was only one criterion (independent variable, factor is HME) for classifying the responses obtained from the experimental results,

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one-way ANOVA was performed. If the ANOVA showed a difference between the treatment groups, Duncan's multiple range test was used to determine whether there was a significant difference in the averages between the treatment groups. Since there were many treatment groups, attention was focused on the various comparisons. The data were expressed in terms of mean values and standard error of the mean (SEM); cases with $P \leq 0.05$ were considered statistically significant. Before conducting the experiments, a preplanned comparison regarding the condition of interest was established. It was unnecessary to adjust the calculated significance probability when considering all comparisons not tested. Orthogonal contrasts are often used for evaluation when comparing means in an experimental design. In the experimental design of this study, this method was not used for the following reasons. Orthogonal contrasts are orthogonal (independent) when the sum of the products of the effects of processing levels for a pair of linear contrasts is zero. The sum of squares of the orthogonal becomes the processing sum of squares. If the preplanned multiple comparison is an orthogonal comparison, then there is no need to correct the significance probability. Orthogonal comparisons are performed when all comparisons are independent and the same mean is not compared twice.

RESULTS AND DISCUSSION

Egg production and egg quality

Egg production, egg quality, feed intake, egg mass, and feed conversion ratio (FCR) of laying hens fed diets containing HME are presented in Table 2 and Table 3, respectively. Egg production, egg weight, egg mass, and feed intake were significantly greater in the HME and PC groups compared to the CON group ($P < 0.05$). However, there were no significant differences ($P > 0.05$) in the egg production parameters between the HME and PC groups (Table 2). FCR showed a tendency to increase by approximately 0.3 points in the HME group compared to the CON group, but there was no significant difference among all treatment groups. This is be-

Table 2. Effect of feeding diets with housefly maggot extract (HME) on egg production, egg weight, feed intake and feed conversion ratio (FCR) in laying hens under a digital livestock system

Attribute	Groups				SEM	*P*-value
	CON	PC	HME100	HME150		
Egg production (%)	88.1[b]	89.8[a]	90.4[a]	89.8[a]	0.443	0.025
Egg weight (g)	61.1[b]	63.8[a]	63.4[a]	64.0[a]	0.257	0.019
Feed intake (g/day)	124[b]	148[a]	150[a]	148[a]	0.456	0.020
Egg mass (g/day)	53.8[b]	57.3[a]	57.3[a]	57.5[a]	0.113	0.010
FCR	2.30	2.58	2.62	2.57	0.050	0.220
Mortality (%)	0	0	0	0	–	–

CON = no added HME; Egg mass = % egg production × egg weight; FCR = feed intake/egg mass; HME100 = HME 100 ppm; HME150 = HME 150 ppm; PC = positive control, 8 ppm of avilamycin

[a,b]Mean values within the same row with no common superscripts differ significantly ($P < 0.05$)

Table 3. Effect of feeding diets with housefly maggot extract (HME) on egg quality in laying hens under a digital livestock system

Attribute	Groups				SEM	*P*-value
	CON	PC	HME100	HME150		
Eggshell thickness (mm)	0.30[b]	0.41[a]	0.42[a]	0.39[a]	0.003	< 0.01
Eggshell strength (kg/cm^2)	2.54[b]	3.97[a]	4.02[a]	3.91[a]	0.015	< 0.01
Albumin height (mm)	7.01[b]	8.52[a]	8.15[a]	8.34[a]	0.035	< 0.01
Haugh unit	81.6[b]	84.0[a]	83.9[a]	84.1[a]	0.345	< 0.01
Egg yolk color	9.01	9.15	9.07	8.89	0.031	0.358

CON = no added HME; HME100 = HME 100 ppm; HME150 = HME 150 ppm; PC = positive control, 8 ppm of avilamycin

[a,b]Mean values within the same row with no common superscripts differ significantly ($P < 0.05$)

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lieved to be because the egg mass (laying rate and egg weight) was higher in the HME group compared to the CON group.

Egg quality factors, such as eggshell thickness, eggshell strength, Haugh unit, and albumin height, were significantly improved in the HME and PC groups than the CON group, but there were no differences between the HME and PC groups (Table 3, $P < 0.05$). The egg yolk color was similar among all groups. The grade of egg yolk for all groups ranged 8.89 to 9.15 points. The present study aimed to determine the feasibility of antibiotic substitution and optimal levels of HME in the diets of laying hens. Dietary feed containing 100–150 ppm of HME increased egg production and greatly improved egg quality to the level achieved by adding avilamycin. Thus, egg production and egg quality were shown to have stable points on plateaus that did not increase further with HME addition above 100 ppm.

The study results indicate that HME as an antibiotic replacement improves egg production and egg quality in laying hens. This further suggests that HME may increase the bioavailability of nutrition (not determined) along with feed intake, thereby increasing egg production. In the future, additional research will be needed regarding the bioavailability of nutrients to poultry fed a diet containing HME. It is well-established that various antimicrobial peptides increase nutrient bioavailability and improve animal productivity (Rodrigues et al. 2022). The findings of this study share the views of Park's (2023) report that feeding 100 ppm of HME improves the growth performances of broiler chickens significantly. Egg quality is a very important factor regarding consumer preferences. Results show that egg quality may be improved by increasing the bioavailability of nutrients (not determined) and animal health through stimulation of immune cell development and function via antimicrobial peptides contained in HME (Rodrigues et al. 2022). It can be considered that the high bioavailability of proteins, amino acids, fats, total energy, calcium, and phosphorus in the HME groups was greater than that in the CON group. Increased animal immunity is critical to maintaining animal health while increasing nutrient bioavailability (De Marco et al. 2015). There is no doubt that HME has great potential as an immunomodulator in poultry via the action mechanisms of antimicrobial peptides without anti-nutritive substances (Makkar et al. 2014). However, the large-scale use of HME in diets involving livestock production systems has many constraints owing to the legal barriers established by the European Union (Regulation EC No. 1069/2009) and production restrictions (Park 2022).

Blood biomarkers

Blood biomarkers related to the immune functions of laying hens fed diets containing HME are shown in Table 4. The blood IgG concentrations in the HME and PC groups increased significantly more than in the CON group ($P < 0.05$). On the other hand, there was no significant difference ($P > 0.05$) in the IgG values between the HME and PC groups. Leukocytes, white blood cells, and heterophil (H) concentrations also showed no significant differences ($P > 0.05$) between the groups. However, the lymphocyte (L) concentration was significantly higher in the HME and PC groups than in the CON group ($P < 0.05$). However, the values were similar between the HME and PC groups. Corticosterone levels and H : L ratios were significantly lower in the HME and PC groups than in the CON group ($P < 0.05$). However, there

Table 4. Effect of feeding diets with housefly maggot extract (HME) on blood biomarkers of immune function in laying hens under a digital livestock system

Attribute	Groups				SEM	*P*-value
	CON	PC	HME100	HME150		
IgG (mg/ml)	2.30[b]	3.18[a]	3.41[a]	3.28[a]	0.021	< 0.01
Corticosterone (ng/ml)	3.74[a]	2.07[b]	1.55[b]	1.68[b]	0.010	< 0.01
White blood cell (K/ul)	7.17	6.51	7.05	6.78	0.038	0.291
Heterophils (H) (%)	15.6	16.0	15.9	15.4	0.094	0.375
Lymphocytes (L) (%)	62.3[b]	73.5[a]	72.9[a]	73.0[a]	0.364	< 0.01
H : L ratios	0.25[a]	0.22[b]	0.22[b]	0.21[b]	0.002	< 0.01

CON = no added HME; HME100 = HME 100 ppm; HME150 = HME 150 ppm; PC = positive control, 8 ppm of avilamycin
[a,b]Mean values within the same row with no common superscripts differ significantly ($P < 0.05$)

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were no significant differences ($P > 0.05$) in the corticosterone levels and H : L ratios between the HME and PC groups.

It has been known that blood parameters reach a plateau and maintain a constant level at three to five weeks (more than 21 days) after feed intake in monogastric animals, including birds (Wang et al. 2022). Therefore, blood biomarkers of immune function were measured from five to 10 weeks (45, 47, and 50 weeks) after evaluating the performance parameters from 40 to 45 weeks. HME greatly improves the level of IgG, an immune-related biomarker, in the blood. This result is also supported by the reports showing that HME contributes to the development of immune cells, thymus, spleen, serum immunoglobulins, IgG, IgA, and IgM in rats and broilers (Park 2023). The high IgG levels in the HME groups are attributable to the increased bifidobacteria (not determined) and *Lactobacillus* in the cecum of laying hens, as expressed by the antimicrobial peptides contained in the HME (Sanchez et al. 2010). Immunoproteins play important roles in the conversion of IgM to IgG, as well as the immune capacity of IgA, which is mainly dependent on the spleen and thymus in poultry (Yu et al. 2021). Leukocytes (white blood cells, lymphocytes, heterophils) refer to cells other than red blood cells in the blood (Elagib and Elzubeir 2012; Genovese et al. 2013; Eyng et al. 2015).

Leukocytes are immune cells that protect animal health by enhancing the body's defence against foreign substances and infections, killing the infected cells, and producing antibodies (Eyng et al. 2015). Heterophils are important cells of the innate immune system that are analogous to mammalian neutrophils and are responsible for phagocytosis and lysis (Genovese et al. 2013). Corticosterone level and H : L ratio are known to be stress indicators in poultry. When poultry are exposed to animal stress, their immune systems are negatively affected by cellular immunity due to the decreased lymphocyte and total white blood cell counts and increased H : L ratio (Elagib and Elzubeir 2012). Gut-beneficial bacteria, such as bifidobacteria and *Lactobacillus*, play important roles in the circulating concentrations of immunoglobulins and nonspecific immune-cell activities of granulocytes. High serum IgG levels in animals fed a diet containing HME indicate the high efficiency of antimicrobial peptides in the extract for increasing humoral immunity. The immune proteins, IgG, that have the highest level in the blood are produced by the B-cells in the bone marrow and are known as biomarkers of humoral immunity (Liu et al. 2008; Park et al. 2010).

Microbiota

Microbiota measured in the faeces of laying hens fed diets containing HME are presented in Table 5. The faecal *Lactobacillus* in the HME and PC groups were significantly higher than in the CON group ($P < 0.05$). There was no significant difference between the HME and PC groups. Microbiota observed in the faeces, namely coliform bacteria, total aerobic bacteria, and *E. coli*, in the HME and PC groups, were significantly lower, respectively, than those in the CON group ($P < 0.05$); however, these faecal microbiota counts were similar between the HME and PC groups.

Research has shown that adding HME to laying hen feed strengthens the *Lactobacillus* population while suppressing the growths of *E. coli* and coliforms; the antimicrobial peptides stimulate the growths of bifidobacteria (not determined) and *Lactobacillus* (Rodrigues et al. 2022). The enhanced bifidobacteria and *Lactobacillus* in the poultry caecum allow the bifidogenic effects to selectively stimu-

Table 5. Effect of feeding diets with housefly maggot extract (HME) on fecal microbiota (cfu/g fresh feces) in laying hens under a digital livestock system

Attribute	Groups				SEM[2]	*P*-value
	CON	PC	HME100	HME150		
Lactobacillus	7.05[b]	8.26[a]	8.81[a]	8.53[a]	0.044	< 0.01
Coliform bacteria	6.27[a]	5.75[b]	5.63[b]	5.45[b]	0.032	< 0.01
Total aerobic bacteria	6.76[a]	6.03[b]	6.07[b]	5.98[b]	0.027	< 0.01
Escherichia coli	6.12[a]	5.51[b]	5.17[b]	5.23[b]	0.021	< 0.01

CON = no added HME; HME100 = HME 100 ppm; HME150 = HME 150 ppm; PC = positive control, 8 ppm of avilamycin
[a,b]Mean values within the same row with no common superscripts differ significantly ($P < 0.05$)

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late the beneficial microbiota, thereby suppressing the growth of harmful microbiota (Jozefiak and Engberg 2017). *Lactobacillus* is the dominant species of microbiota in the guts of animals that can inhibit the growth of pathogens by maintaining microbiota balance and an acidic environment in the guts of poultry (Malematja et al. 2023). The higher counts of faecal microbiota *Lactobacillus,* as well as lower amounts of coliforms, total aerobic bacteria, and *E. coli* in the HME groups, may also be attributed to the action mechanisms of the antimicrobial peptides in HME (Park et al. 2010; Makkar et al. 2014). The antimicrobial peptides are known to stimulate the growths of bifidobacteria and *Lactobacillus* by destroying the transmembrane potentials of harmful bacteria, thus enhancing immunity (Cheng et al. 2014; Rodrigues et al. 2022; Malematja et al. 2023). The bifidogenic effects of selectively stimulating the growths of bifidobacteria and *Lactobacillus*, which are beneficial to animal health, further inhibit the growth of harmful strains (Park et al. 2010). The high IgG levels in the HME groups and their increased counts of faecal *Lactobacillus* compared to the CON group were considered to be a form of bifidogenic effect from the antimicrobial peptides in the HME. Beneficial bacteria such as bifidobacteria and *Lactobacillus* in the gut of animals play important roles in the biosynthesis of fermentation products that supply the necessary energy to the intestinal epithelial cells, immune system, and vitamin K synthesis while preventing the adhesion of pathogens in the digestive tract (Tako et al. 2008). Bifidobacteria and *Lactobacillus* secrete an antimicrobial substance called bacteriocin as well as organic acids, such as lactic acid and acetic acid, to suppress the growth of harmful bacteria in the digestive tracts of animals (Qui 2023). However, this study does not entail the determination of the major organic acids produced by bifidobacteria and *Lactobacillus*. Hence, additional research is needed in this regard. In Park's (2023) report on broiler chickens, the HME group had significantly higher concentrations of acetic acid, propionic acid, and total short-chain fatty acids but lower levels of butyric acid, isobutyric acid, valeric acid, and isovaleric acid in the poultry gut. This supports the present results, showing that HME plays a key role in maintaining the balance of the poultry cecal microbiota. The *Lactobacillus* counts in the HME groups increased significantly more than in the CON group. The increased bifidobacteria and *Lactobacillus* may improve immunity by reducing harmful enteric bacteria and stimulating the immune system (Park et al. 2017; Park 2022). The increased counts of beneficial *Lactobacillus* and greatly decreased harmful bacterial counts in the HME groups were attributed to these mechanisms.

CONCLUSION

The results of this study show that HME acts as an antibiotic alternative to greatly improve egg production, egg quality (eggshell thickness, eggshell strength, Haugh unit, and albumin height), blood biomarkers of immune function, and microbiota population in laying hens under a digital livestock system. These findings suggest that adding 100 ppm of HME to poultry feed could be an effective substitute for antibiotics. It can improve egg production and quality via stimulated immune functions and balanced cecal microbiota in laying hens under a digital livestock system.

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Conflict of interest

The authors declare no conflict of interest.

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