




# Effects of housefly larvae meal and multi-enzyme-probiotic supplementation on performance, egg quality, and blood biochemistry in laying hens

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## ABSTRACT

This study investigated the effects of dietary housefly larvae meal (HFLM) and Rayabold (multi-enzyme-probiotic supplement) on the performance, egg quality, and blood biochemistry of 192 Lohmann LSL-Lite laying hens at 56 weeks of age. A 10-week feeding trial was conducted using a  $4 \times 2$  factorial design, with four HFLM inclusion levels (0, 5, 10, and 15 %) and two Rayabold levels (0 and 0.05 %). The inclusion of HFLM, with or without Rayabold, significantly reduced feed intake compared to the control; however, Rayabold partially offset this reduction at the 5 % and 10 % HFLM levels. Although feed intake was reduced, egg production remained unaffected, while egg weight increased notably in hens receiving 15 % HFLM combined with Rayabold. Both 10 % and 15 % inclusion levels of HFLM improved feed conversion ratio, protein efficiency, and energy efficiency, with Rayabold further enhanced protein and energy utilization at the 15 % inclusion level. Eggshell weight, thickness, and yolk height improved significantly in the 15 % HFLM + Rayabold group. Metabolic responses included reduced blood glucose at 5 % and 10 % HFLM, and lower cholesterol at 10 %. Furthermore, triglyceride levels declined with both HFLM and Rayabold supplementation. Aspartate aminotransferase activity decreased in the 10 % and 15 % HFLM groups, while alanine aminotransferase was highest in the 15 % + Rayabold group, suggesting possible hepatic stress. Overall, 10 % inclusion of HFLM, with or without Rayabold, offers a nutritionally effective and metabolically safe strategy to enhance laying hen productivity and physiological health, supporting its potential as a sustainable alternative protein source.

## 1. Introduction

The poultry industry is crucial for global food security, supplying a significant portion of the world's animal protein, primarily as chicken meat and eggs (Daghir et al., 2021). However, the increasing cost and scarcity of traditional protein sources like soybean and fishmeal necessitate the exploration of sustainable alternatives (Hussein et al., 2017). Insect-based feed has emerged as a promising solution, attracting considerable interest due to its nutritional richness and environmental sustainability (van Huis, 2021). Insects offer a balanced profile of protein, fats, vitamins, and minerals, making them suitable for animal feed (Gasco et al., 2019; Belhadj Slimen et al., 2023). Furthermore, insect production requires fewer resources (land, water, and feed) and can

utilize organic waste, reducing the environmental impact of animal agriculture (Gasco et al., 2019; Sorjonen et al., 2019; Belhadj Slimen et al., 2023).

Among these, housefly larvae (*Musca domestica*) are particularly attractive for poultry diets due to their high protein and fat content, favorable amino acid composition, and abundance of micronutrients (Hussein et al., 2017; Belhadj Slimen et al., 2023). Although several studies have examined insect meals in poultry nutrition (Khan et al., 2018; Liu et al., 2021; Sedgh-Gooya et al., 2021), research focusing specifically on housefly larvae meal (HFLM) in layer diets remains limited (Agunbiade et al., 2007; Zammit and Park, 2024). While HFLM shows potential to replace conventional feedstuffs, determining optimal inclusion levels is essential to avoid negative effects on nutrient

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digestibility or egg quality, particularly shell integrity (Agunbiade et al., 2007). In addition to nutritional value, insect-based feeds may offer functional benefits due to compounds such as chitin and antimicrobial peptides (AMPs) (Belhadj Slimen et al., 2023). Although animals generally lack chitinase, microbial fermentation of chitin in the gut may provide prebiotic effects and immune modulation (Józefiak et al., 2016; Elieh et al., 2018; de Carvalho et al., 2019). However, the indigestibility of chitin can also act as an anti-nutritional factor, potentially limiting nutrient availability.

To improve nutrient absorption and mitigate such limitations, feed additives like enzymes, probiotics, and prebiotics are increasingly used in poultry diets (Slominski, 2011; Al-Khalaifah, 2018; Haider et al., 2024). Rayabold, a multi-functional feed supplement combining probiotics, prebiotics, and enzymes (Khodaei et al., 2022; Salehizadeh et al., 2025), is designed to enhance gut health and feed utilization efficiency.

Therefore, the objective of this study was to evaluate the effects of dietary HFLM, with or without Rayabold supplementation, on productive performance, egg quality, and blood biochemical parameters in laying hens. We hypothesized that (1) inclusion of HFLM at appropriate levels would improve feed efficiency and metabolic health without compromising egg production (EP) or quality, and (2) combining HFLM with Rayabold would further enhance these effects by improving nutrient utilization and mitigating potential anti-nutritional impacts of insect meal.

## 2. Materials and methods

### 2.1. Ethics statement

The animal care procedures followed in the experiment were approved by the ethics committee of the Razi University in Kermanshah, Iran (IR.RAZI.AEC.1404.014).

### 2.2. Experimental design and diets

A feeding trial was conducted using 192 Lohmann LSL-Lite laying hens at 56 weeks of age. Hens were housed in cages at a density of six birds per replicate, with eight dietary treatments evaluated across four replicates per treatment. The 10-week trial was conducted under controlled environmental conditions, maintaining a 16:8 h light-dark photoperiod (16L:8D), a temperature range of 18–21°C, and a relative humidity of 30–40%. Feed was provided daily at a rate of approximately 120 g per hen, and water was available ad libitum. The experimental treatments consisted of four dietary HFLM concentrations (0, 5, 10, and 15%) and two Rayabold supplement concentrations (0 and 0.05%), arranged in a 4 × 2 factorial design (Table 1). Table 2 presents the full formulation, including ingredient and chemical composition of the

**Table 1**  
Experimental treatments.

Treatments	Housefly larvae (%)	Rayabold supplement <sup>1</sup> (%)
1	0	0
2	5	0
3	10	0
4	15	0
5	0	0.05
6	5	0.05
7	10	0.05
8	15	0.05

<sup>1</sup> Rayabold is a commercial supplement, which contains a blend of probiotics (*Lactobacillus plantarum*, *L. delbrueckii*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus salivarius*, *L. casei*, *L. rhamnosus*, *Bacillus coagulans*, *B. subtilis*, *B. licheniformis*, and *L. acidophilus*), prebiotics (autolyzed yeast, mannan oligosaccharide, β-glucan, and inulin), and a suite of enzymes (protease, lipase, glucose oxidase, hemicellulase, α-amylase, β-amylase, xylanase, β-glucanase, and phytase).

**Table 2**  
Ingredients and composition of experimental diets (%).

Diets				
Ingredients	Control	HFLM5	HFLM10	HFLM15
Corn	59.20	57.99	56.89	55.58
Soybean meal	27.50	21.00	13.76	7.12
Wheat bran	0	3.00	6.00	6.50
Calcium carbonate	10.80	10.92	11.01	11.07
HFLM <sup>1</sup>	0	5.00	10.00	15.00
Bentonite	0	0	0.50	3.00
Dicalcium phosphate	1.00	0.66	0.32	0.02
Multi mix <sup>2</sup>	0.50	0.50	0.50	0.50
Rayabold <sup>3</sup>	0.05	0.05	0.05	0.05
Binder toxin	0.10	0.10	0.10	0.10
DL-methionine	0.15	0.08	0	0
L-lysine	0	0	0.17	0.36
Salt	0.20	0.20	0.20	0.20
Oil	0.50	0.50	0.50	0.50
Total	100	100	100	100
<b>Calculated composition</b>				
ME (kcal/kg)	2700	2700	2700	2700
Crude protein (%)	17.00	17.00	17.00	17.00
Crude fiber (%)	3.14	2.90	2.68	2.23
Calcium (%)	4.20	4.20	4.20	4.20
Available phosphorus (%)	0.42	0.42	0.42	0.42
Lysine (%)	0.81	0.68	0.65	0.65
Methionine + Cysteine (%)	0.63	0.66	0.66	0.76
Threonine (%)	0.60	0.67	0.73	0.79
Tryptophan (%)	0.20	0.18	0.15	0.12
Arginine (%)	1.10	1.12	1.11	1.1
Isoleucine (%)	0.69	0.61	0.51	0.41
Valine (%)	0.77	0.73	0.66	0.6

<sup>1</sup> HFLM = Housefly larvae meal (5, 10, 15%); ME = Metabolizable energy.

<sup>2</sup> Vitamin mixture per 0.25 kg/100 kg of diet: Vitamin A, 7,700,000 IU; Vitamin D3, 3,300,000 IU; Vitamin E, 6600 mg; VitaminK3, 550 mg; thiamine, 2200 mg; riboflavin, 4400 mg; Vitamin B6, 4400 mg; capantothenate, 550 mg; nicotinic acid, 200 mg; folic acid, 110 mg; choline chloride, 275,000 mg; biotin, 55 mg; Vitamin B12, 8.8 mg. <sup>b</sup>Mineral mixture per 0.25 kg/100 kg of diet: Mn, 66,000 mg; Zn, 66,000 mg; Fe, 33,000 mg; Cu, 8,800 mg; Se, 300 mg.

<sup>3</sup> All diets were combined with or without Rayabold supplement (a blend of probiotics (*Lactobacillus plantarum*, *L. delbrueckii*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus salivarius*, *L. casei*, *L. rhamnosus*, *Bacillus coagulans*, *B. subtilis*, *B. licheniformis*, and *L. acidophilus*), prebiotics (autolyzed yeast, mannan oligosaccharide, β-glucan, and inulin), and a suite of enzymes (protease, lipase, glucose oxidase, hemicellulase, α-amylase, β-amylase, xylanase, β-glucanase, and phytase).

experimental diets, formulated to meet the established nutrient requirements of Lohmann LSL-Lite hens (Lohmann Breeders, 2020). Housefly larvae was sourced from Fardad Kian Fartak Company (Iran), where mature flies were reared and allowed to oviposit on a plant-based substrate. The resulting larvae were then separated and subjected to proximate analysis by the supplier's laboratory, which reported a composition of 53% crude protein, 20% crude fat, and 8% crude fiber. To potentially enhance nutrient utilization from the HFLM, diets were supplemented with a commercially available multi-component product (Rayabold's supplement, Fardad Kian Fartak Company, Iran). This supplement contained a blend of probiotics (*Lactobacillus plantarum*, *L. delbrueckii*, *Enterococcus faecium*, *casei*, *L. rhamnosus*, *Bifidobacterium bifidum*, *Bacillus coagulans*, *B. subtilis*, *B. licheniformis*, *Streptococcus salivarius*, *L. and L. acidophilus*), prebiotics (autolyzed yeast, mannan oligosaccharide, β-glucan, and inulin), and a suite of enzymes (protease, lipase, glucose oxidase, hemicellulase, α-amylase, β-amylase, xylanase, β-glucanase, and phytase). Sample size was determined based on expected effect sizes from previous studies to achieve 80% power at α = 0.05.

### 2.3. Productive performance

Feed intake (FI) was quantified by subtracting residual feed at the

end of each week from the daily feed allocation. Eggs were collected and weighed daily. The ratio of mean FI (g) to mean egg weight (EW, g) yielded the feed conversion ratio (FCR). Mortality was recorded throughout the experimental period to facilitate data adjustments. The protein efficiency ratio (PER) was determined by dividing EW (g) by the protein intake (g), with the latter derived from the product of total FI and the feed's protein content. To determine the energy efficiency ratio (EER), the total weight of eggs produced was divided by the metabolizable energy (ME) intake. ME intake was calculated as the product of total FI and feed energy content.

#### 2.4. Egg quality traits

Egg quality assessment occurred after the experiment's conclusion, with daily sampling of one egg per replicate over a three-day period. The analyzed parameters included yolk weight, albumen weight, yolk height, albumen height, shell weight, shell thickness, and Haugh unit. The ratio of shell weight to total EW was used to determine shell percentage. Eggshell thickness was determined by averaging measurements taken with a micrometer at three locations on each egg: the large end, the middle (equator), and the small end (pointed end). This triplicate measurement accounts for potential variations in shell thickness across the egg's surface. Yolk height was precisely measured using a tripod micrometer (0.01 mm accuracy; Mitutoyo, Japan). The Haugh unit method (Eisen et al., 1962) was employed to determine albumen quality. This method uses the formula:  $100 \times \log(H+7.6 - 1.7W^{0.37})$ , where H is albumen height (mm) and W is EW (g).

#### 2.5. Blood biochemical parameters

At the conclusion of the experiment, one bird from each replicate was sampled for blood via the brachial vein. Serum biochemical parameters, including glucose, cholesterol, triglycerides, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were subsequently analyzed. Serum samples, obtained by centrifugation ( $1008 \times g$ , 15 min), were stored at  $-20^\circ\text{C}$  and later analyzed using commercial kits (Pars Azmun, Tehran, Iran).

#### 2.6. Statistical analysis

All data were subjected to statistical analysis. This included a normality test using the Shapiro-Wilk method, followed by a two-way ANOVA based on a completely randomized  $4 \times 2$  factorial design. This design examined the main effects of four levels of HFLM and two levels of Rayabold supplement, as well as their interaction. The statistical model can be represented as:  $Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$ . The model incorporated main effects for HFLM ( $A_i$ ) and Rayabold ( $B_j$ ), as well as an interaction term ( $AB_{ij}$ ), with the observed value represented by  $Y_{ijk}$ , the overall mean by  $\mu$ , and the random error by  $e_{ijk}$ . All analyses were performed using the general linear model (GLM) procedure in SAS 9.4 software (SAS, 2015). Mean comparisons were performed using Duncan's multiple range test ( $P < 0.05$ ).

### 3. Result

#### 3.1. Productive performance

The effects of HFLM and Rayabold supplementation on laying hen productive performance are presented in Table 3. While experimental treatments exerted no significant influence on EP ( $P > 0.05$ ), a significant interaction effect was observed on FI, EW, FCR, PER, and EER ( $P < 0.05$ ). Dietary inclusion of 5 %, 10 %, and 15 % HFLM, both with and without Rayabold supplementation, significantly decreased FI than the control diet and the diet containing Rayabold alone ( $P < 0.05$ ). However, Rayabold supplementation in the 5 % and 10 % HFLM treatments significantly increased FI compared to the respective HFLM treatments without enzyme ( $P < 0.05$ ). Regarding EW, the highest value was observed in the 15 % HFLM treatment with 0.05 % Rayabold, which was significantly different from all other treatments except the enzyme-only treatment and the 10 % HFLM treatment ( $P < 0.05$ ). A statistically significant improvement in FCR ( $P < 0.05$ ) was achieved when diets contained either 10 % or 15 % HFLM, irrespective of Rayabold supplementation, relative to the remaining treatment groups. Furthermore, the highest PER and EER values were observed in the 15 % and 10 % HFLM treatments, respectively, both with and without Rayabold supplementation ( $P < 0.05$ ). Rayabold supplementation in the 15 % HFLM

**Table 3**  
Influence of dietary housefly larvae meal and Rayabold supplement on productive performance of laying hens.

Treatments	FI (g/h/day)	HDEP (%)	EW (g)	FCR (g/g)	PER	EER	
HFLM (%)							
Control	119.82 <sup>a</sup>	89.68	64.97	1.85 <sup>a</sup>	3.19 <sup>c</sup>	0.20 <sup>c</sup>	
5	116.61 <sup>b</sup>	89.22	63.76	1.84 <sup>a</sup>	3.22 <sup>c</sup>	0.20 <sup>c</sup>	
10	107.60 <sup>c</sup>	95.00	64.22	1.68 <sup>b</sup>	3.51 <sup>b</sup>	0.22 <sup>b</sup>	
15	104.02 <sup>d</sup>	92.19	65.83	1.59 <sup>c</sup>	3.72 <sup>a</sup>	0.23 <sup>a</sup>	
Rayabold (%)							
0	111.92	90.62	63.92	1.76	3.37	0.21	
0.05	112.10	92.42	65.47	1.71	3.45	0.22	
Interactions							
HFLM		Rayabold					
0	0	119.79 <sup>a</sup>	89.69	63.56 <sup>b</sup>	1.88 <sup>a</sup>	3.12 <sup>d</sup>	0.20 <sup>d</sup>
5	0	114.85 <sup>c</sup>	84.38	64.27 <sup>b</sup>	1.81 <sup>ab</sup>	3.29 <sup>cd</sup>	0.21 <sup>cd</sup>
10	0	110.15 <sup>d</sup>	95.94	64.98 <sup>ab</sup>	1.70 <sup>bc</sup>	3.47 <sup>bc</sup>	0.22 <sup>bc</sup>
15	0	102.90 <sup>f</sup>	92.50	62.89 <sup>b</sup>	1.64 <sup>cd</sup>	3.59 <sup>b</sup>	0.23 <sup>b</sup>
0	0.05	119.85 <sup>a</sup>	89.69	66.40 <sup>ab</sup>	1.81 <sup>ab</sup>	3.26 <sup>cd</sup>	0.21 <sup>cd</sup>
5	0.05	118.38 <sup>b</sup>	94.06	63.25 <sup>b</sup>	1.88 <sup>a</sup>	3.14 <sup>d</sup>	0.20 <sup>d</sup>
10	0.05	105.04 <sup>e</sup>	94.06	63.45 <sup>b</sup>	1.66 <sup>c</sup>	3.55 <sup>b</sup>	0.22 <sup>b</sup>
15	0.05	105.15 <sup>e</sup>	91.88	68.77 <sup>a</sup>	1.53 <sup>d</sup>	3.85 <sup>a</sup>	0.24 <sup>a</sup>
SEM	0.715	7.66	3.86	0.114	0.205	0.013	
P value							
HFLM	0.000	0.136	0.456	0.000	0.000	0.000	
Rayabold	0.319	0.352	0.115	0.164	0.120	0.076	
HFLM*Rayabold	0.000	0.135	0.028	0.041	0.038	0.050	

HFLM = Housefly larvae meal; FI = Feed intake; HDEP = Hen-day egg production; FCR = Feed conversion ratio; PER = Protein efficiency ratio; EER = Energy efficiency ratio. Rayabold = Supplement containing prebiotic, probiotic, and multi-enzyme. SEM = Standard error of mean. Means were calculated on  $n = 4$  replicates (6 birds per replicate) per treatment. All data were analyzed by two-way ANOVA based on a completely randomized  $4 \times 2$  factorial design. Mean comparisons were conducted using Duncan's multiple range test ( $P < 0.05$ ). Means with different superscripts in columns differ significantly.

treatment further significantly increased PER and EER compared to the unsupplemented 15 % HFLM treatment ( $P < 0.05$ ).

### 3.2. Egg quality traits

Table 4 presents the impact of dietary HFLM and Rayabold supplementation on laying hen egg quality parameters. No significant impact of the different treatments were observed on EW, albumen weight, albumen height, yolk weight, or Haugh units ( $P > 0.05$ ). However, a significant interaction between HFLM and Rayabold treatments was detected for eggshell weight, eggshell thickness, and yolk height ( $P < 0.05$ ). While no dietary treatment significantly differed from the control group in eggshell weight, hens fed 10 % HFLM with Rayabold exhibited the lowest eggshell weight, which was significantly lower than those fed 15 % HFLM with Rayabold and the Rayabold-only treatment, which exhibited the highest ( $P < 0.05$ ). Regarding eggshell thickness, the 10 % HFLM, Rayabold-only, and 15 % HFLM with Rayabold treatments resulted in significantly greater eggshell thickness than the control group ( $P < 0.05$ ). Notably, the inclusion of Rayabold into 15 % HFLM treatment significantly increased eggshell thickness ( $P < 0.05$ ). Furthermore, the Rayabold-only and 15 % HFLM with Rayabold treatments significantly increased yolk height relative to the control ( $P < 0.05$ ).

### 3.3. Blood parameters

Table 5 presents the effects of HFLM and Rayabold supplementation on laying hen blood parameters. Blood glucose and cholesterol concentrations were significantly altered by HFLM, as shown by statistical analysis ( $P < 0.05$ ). Specifically, dietary inclusion of 5 % and 10 % HFLM significantly reduced blood glucose concentrations compared to both the control group and the 15 % HFLM treatment ( $P < 0.05$ ). Regarding cholesterol, hens fed the control diet and the 10 % HFLM diet exhibited significantly lower concentrations compared to those receiving 5 % and 15 % HFLM ( $P < 0.05$ ). Significant interaction effects between the experimental treatments were observed for triglyceride, AST, ALT, and ALP concentrations ( $P < 0.05$ ). The combined dietary administration of HFLM and Rayabold led to a statistically significant

decrease in triglyceride concentrations relative to the control group ( $P < 0.05$ ). Furthermore, Rayabold supplementation in the 5 % and 15 % HFLM diets further decreased triglyceride content compared to their unsupplemented counterparts. Hens fed 10 % and 15 % HFLM, both with and without Rayabold, exhibited significantly lower AST levels than other treatment groups ( $P < 0.05$ ). For ALT, the highest concentrations were observed in the 15 % HFLM treatment supplemented with Rayabold, while the lowest were found in the control group. ALP concentrations were highest in the control group, differing significantly from all other treatments, with the lowest concentration observed in the 5 % HFLM treatment ( $P < 0.05$ ). The experimental treatments had no discernible impact on blood albumin concentration ( $P > 0.05$ ).

## 4. Discussion

### 4.1. Productive performance

This study demonstrated that the inclusion of HFLM in laying hen diets significantly reduced FI, particularly at 10 % and 15 % inclusion levels, compared to the control. These findings align with earlier reports where insect-based meals such as maggot meal led to decreased FI in poultry (Okah & Onwujiariri, 2012; Khan et al., 2016; Khan et al., 2018; Salehizadeh et al., 2025). The reduction in FI is plausibly attributed to the higher nutrient density and superior digestibility of HFLM relative to conventional protein sources such as soybean meal or fishmeal (Novodvorski et al., 2023). Insect meals typically contain a high concentration of digestible protein, energy-rich fats, and bioavailable minerals, allowing birds to fulfill their nutritional requirements with reduced feed volume (Belhadj Slimen et al., 2023). Moreover, high chitin content may also contribute to early satiety, thereby limiting FI at elevated inclusion levels (Belhadj Slimen et al., 2023). Feed palatability and visual characteristics may also influence intake (Khosravinia, 2007). Dark pigmentation associated with insect meals—particularly at higher levels—has been suggested to reduce feed attractiveness, potentially discouraging consumption (Khosravinia, 2007; Tabekkh, 2015). Similar reductions in FI have been documented in diets containing black soldier fly larvae meal (BSFLM) at higher inclusion levels (Marono et al., 2017; Bovera et al., 2018). However, discrepancies in the literature remain.

**Table 4**  
Influence of dietary housefly larvae meal and Rayabold supplement on egg quality traits of laying hens.

Treatments	EW (g)	Yolk W (g)	Albumen W (g)	Shell W (g)	Shell T ( $10^{-2}$ mm)	Yolk H (mm)	Albumen H (mm)	HU	
HFLM (%)									
Control	65.44	18.09	37.18	10.16	0.375	15.98	3.70	47.83	
5	64.22	18.10	36.78	9.32	0.378	15.62	3.46	46.21	
10	64.68	18.26	36.90	9.51	0.387	15.47	4.26	57.44	
15	66.30	19.35	37.02	9.92	0.367	16.25	4.18	53.92	
Rayabold (%)									
0	64.38	18.70	36.00	9.68	0.37	15.45 <sup>b</sup>	3.97	53.17	
0.05	65.94	18.20	37.94	9.79	0.38	16.21 <sup>a</sup>	3.83	49.52	
Interactions									
HFLM		Rayabold							
0	0	64.02	17.64	36.29	10.09 <sup>abc</sup>	0.352 <sup>bc</sup>	15.13 <sup>b</sup>	3.82	49.48
5	0	64.73	18.51	37.07	9.15 <sup>bc</sup>	0.383 <sup>ab</sup>	15.77 <sup>ab</sup>	3.14	43.56
10	0	65.45	19.24	36.05	10.16 <sup>abc</sup>	0.405 <sup>a</sup>	15.02 <sup>b</sup>	4.28	58.05
15	0	63.35	19.42	34.61	9.31 <sup>abc</sup>	0.342 <sup>c</sup>	15.89 <sup>ab</sup>	4.62	61.60
0	0.05	66.88	18.56	38.08	10.24 <sup>ab</sup>	0.400 <sup>a</sup>	16.84 <sup>a</sup>	3.58	46.19
5	0.05	63.71	17.71	36.50	9.49 <sup>abc</sup>	0.373 <sup>abc</sup>	15.46 <sup>ab</sup>	3.79	48.86
10	0.05	63.91	17.28	37.76	8.87 <sup>c</sup>	0.368 <sup>abc</sup>	15.93 <sup>ab</sup>	4.23	56.82
15	0.05	69.26	19.29	39.43	10.54 <sup>a</sup>	0.392 <sup>a</sup>	16.61 <sup>a</sup>	3.74	46.24
SEM	4.39	1.34	3.74	0.986	0.029	0.827	1.32	16.99	
P value									
HFLM		0.670	0.077	0.994	0.164	0.427	0.106	0.401	0.344
Rayabold		0.237	0.210	0.075	0.702	0.135	0.003	0.732	0.461
HFLM*Rayabold		0.142	0.078	0.367	0.028	0.000	0.040	0.573	0.516

HFLM = Housefly larvae meal; EW = Egg weight; W = Weight; H = Height; HU = Haugh unit. Rayabold = Supplement containing prebiotic, probiotic and multi-enzyme. SEM = Standard error of mean. Means were calculated on  $n = 4$  replicates (3 eggs per replicate) per treatment. All data were analyzed by two-way ANOVA based on a completely randomized  $4 \times 2$  factorial design. Mean comparisons were conducted using Duncan's multiple range test ( $P < 0.05$ ). Means with different superscripts in columns differ significantly.

**Table 5**  
Influence of dietary housefly larvae meal and Rayabold supplement on blood parameters of laying hens.

Treatments	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)	AST (UI/L)	ALT (UI/L)	ALP (UI/L)	Albumin (g/dL)	
<b>HFLM (%)</b>								
Control	166.13 <sup>a</sup>	130.75 <sup>b</sup>	1126.50 <sup>a</sup>	183.00 <sup>a</sup>	24.37 <sup>c</sup>	705.25 <sup>a</sup>	2.26	
5	151.38 <sup>b</sup>	172.13 <sup>a</sup>	1098.63 <sup>b</sup>	177.13 <sup>a</sup>	27.50 <sup>b</sup>	295.00 <sup>c</sup>	2.40	
10	150.63 <sup>b</sup>	135.13 <sup>b</sup>	1119.63 <sup>a</sup>	165.50 <sup>b</sup>	27.87 <sup>b</sup>	554.75 <sup>b</sup>	2.31	
15	167.13 <sup>a</sup>	178.38 <sup>a</sup>	1096.75 <sup>b</sup>	143.75 <sup>c</sup>	33.00 <sup>a</sup>	545.88 <sup>b</sup>	2.41	
<b>Rayabold (%)</b>								
0	159.50	141.94	1125.31 <sup>a</sup>	161.38 <sup>b</sup>	25.93 <sup>b</sup>	535.38	2.36	
0.05	158.13	166.25	1095.44 <sup>b</sup>	173.31 <sup>a</sup>	30.44 <sup>a</sup>	515.06	2.34	
<b>Interactions</b>								
<b>HFLM</b>								
<b>Rayabold</b>								
0	0	163.75	120.25	1151.25 <sup>a</sup>	179.75 <sup>ab</sup>	21.75 <sup>e</sup>	988.75 <sup>a</sup>	2.30
5	0	155.00	147.50	1116.00 <sup>b</sup>	179.50 <sup>ab</sup>	25.50 <sup>cd</sup>	187.5 <sup>d</sup>	2.38
10	0	159.25	140.00	1119.25 <sup>b</sup>	161.25 <sup>c</sup>	32.25 <sup>b</sup>	406.25 <sup>c</sup>	2.33
15	0	160.00	160.00	1114.75 <sup>b</sup>	125.00 <sup>d</sup>	24.25 <sup>d</sup>	559.00 <sup>bc</sup>	2.43
0	0.05	168.50	141.25	1101.75 <sup>b</sup>	186.25 <sup>a</sup>	27.00 <sup>cd</sup>	421.75 <sup>c</sup>	2.23
5 %	0.05	147.75	196.75	1081.25 <sup>c</sup>	174.75 <sup>ab</sup>	29.5 <sup>bc</sup>	402.5 <sup>c</sup>	2.43
10 %	0.05	142.00	130.25	1120.00 <sup>b</sup>	169.75 <sup>bc</sup>	23.5 <sup>d</sup>	703.25 <sup>b</sup>	2.30
15 %	0.05	174.25	196.75	1078.75 <sup>c</sup>	162.50 <sup>c</sup>	41.75 <sup>a</sup>	532.75 <sup>bc</sup>	2.40
SEM		12.15	33.47	13.45	7.37	2.93	114.65	0.122
<b>P value</b>								
HFLM		0.013	0.014	0.000	0.000	0.000	0.000	0.064
Rayabold		0.751	0.058	0.000	0.058	0.000	0.620	0.668
HFLM*Rayabold		0.078	0.345	0.000	0.000	0.000	0.000	0.784

HFLM = Housefly larvae meal; AST= Aspartate aminotransferase; ALT= Alanine aminotransferase; ALP= Alkaline phosphatase (ALP). Rayabold= Supplement containing prebiotic, probiotic and multi-enzyme. SEM= Standard error of mean. Means were calculated on n = 4 replicates (one sample per replicate) per treatment. All data were analyzed by two-way ANOVA based on a completely randomized 4 × 2 factorial design. Mean comparisons were conducted using Duncan's multiple range test ( $P < 0.05$ ). Means with different superscripts in columns differ significantly.

While some studies reported increased or unchanged FI with maggot or HFLM inclusion (Agunbiade et al., 2007; Hamani et al., 2022; Zammit & Park, 2024), the divergent outcomes may reflect differences in insect species, processing methods (e.g., defatted vs. full-fat, dried vs. fresh), dietary inclusion thresholds, or strain-specific variations in poultry response.

Importantly, the current findings also revealed that supplementation with Rayabold—an enzyme-probiotic complex—significantly improved FI in birds fed 5 % and 15 % HFLM diets. This enhancement likely results from the synergistic action of Rayabold's probiotic, prebiotic, and enzymatic components, which improve gastrointestinal function and feed utilization. Previous studies have reported increased FI with the use of probiotics and prebiotics in poultry diets, potentially through enhanced gut health, stimulation of appetite-regulating hormones, or improved feed palatability (Tang et al., 2017; Rehman et al., 2020; Wang et al., 2024). Additionally, exogenous enzymes may facilitate the breakdown of recalcitrant components such as chitin and non-starch polysaccharides (NSPs), thereby improving nutrient release and absorption (Musigwa et al., 2021; Salehizadeh et al., 2025). The inclusion of Rayabold may have alleviated digestive limitations imposed by insect meal components, restoring or even enhancing FI in enzyme-supplemented groups.

Despite reductions in FI, EP remained statistically unaffected, demonstrating the capacity of HFLM to sustain laying performance. More notably, EW increased in the 15 % HFLM group supplemented with Rayabold, suggesting improved nutrient utilization and partitioning toward egg mass. This is further corroborated by significant improvements in FCR, observed at both 10 % and 15 % HFLM inclusion levels, irrespective of enzyme supplementation. These findings are consistent with earlier reports that highlight insect meals—particularly housefly larvae—as efficient protein sources capable of enhancing nutrient digestibility and utilization in poultry (Belhadj Slimen et al., 2023). Insect-derived nutrients, including AMPs and short-chain fatty acids, are known to support gut integrity and metabolic efficiency, thereby reducing feed requirements without compromising output (Novodvorski et al., 2023). The observed improvements in FCR are particularly noteworthy considering the hens' advanced age (56 weeks), a stage at which feed efficiency typically declines (Belhadj Slimen et al.,

2023). The high-quality amino acid profile of HFLM—comparable to that of fishmeal—may have played a pivotal role in supporting protein deposition and egg formation. These findings are consistent with prior studies reporting enhanced EW and EP in layers fed HFLM (Okah & Onwujiariri, 2012; Zammit & Park, 2024), although some variation persists in the literature depending on the inclusion rate and bird genotype (Agunbiade et al., 2007).

Further, this study demonstrated significant enhancements in PER and EER with 10 % and 15 % HFLM inclusion, with the most pronounced effects seen when Rayabold was included. These results suggest improved metabolic efficiency and nutrient absorption, likely facilitated by the digestibility of medium-chain fatty acids in HFLM (e.g., palmitoleic, oleic acids) and its favorable amino acid composition (da-Silva et al., 2024). These findings are in line with Salehizadeh et al., (2025), who reported enhanced PER and EER in hens fed diets containing housefly larvae and Rayabold, particularly at 15 % inclusion. In contrast, studies involving BSFLM have shown inconsistent effects on nutrient efficiency—Nampijja et al., (2023) reported reduced nutrient utilization, possibly due to high chitin levels and low palatability. Such discrepancies underscore the importance of insect species, processing method, and additive synergy in modulating feed efficiency outcomes.

#### 4.2. Egg quality traits

Consistent with previous findings (Agunbiade et al., 2007; Dörper et al., 2024), the present study found no significant differences in EW, albumen weight, albumen height, Haugh unit scores, or yolk weight across the majority of dietary treatments, including HFLM inclusion levels up to 15 %. These results indicate that HFLM, even at relatively high dietary inclusion, does not negatively impact key internal egg quality traits. From a commercial standpoint, the maintenance of internal egg quality is a critical benchmark, as it ensures that alternative protein sources such as HFLM do not compromise consumer-expected standards or grading specifications (Abebe et al., 2023).

In contrast, significant interaction effects between HFLM inclusion and Rayabold supplementation were observed for eggshell quality traits—specifically shell weight and thickness. Although no individual treatment significantly surpassed the control in terms of shell weight

alone, the 15 % HFLM + Rayabold group exhibited the highest shell weight, which was significantly greater than that of the 10 % HFLM + Rayabold and 5 % HFLM without enzyme groups. These results suggest a synergistic effect between higher HFLM levels and functional enzyme–probiotic supplementation. Such synergy may reflect enhanced bioavailability of key minerals involved in shell formation, particularly calcium and phosphorus (Wang et al., 2024). Housefly larvae are known to contain appreciable levels of both calcium (~0.5 %) and phosphorus (~1.1 %) (Husseini et al., 2017), and these minerals may become more bioavailable in the presence of exogenous enzymes, which can degrade anti-nutritional factors (e.g., phytate or chitin) that otherwise impede mineral absorption (Huang et al., 2024). Moreover, the prebiotic and probiotic components of Rayabold may support mineral retention through gut microbiota modulation, acidification of the intestinal environment, and improved mucosal barrier integrity (Xu et al., 2023; Wang et al., 2024).

Interestingly, although 15 % HFLM + Rayabold achieved the highest eggshell weight, significant improvements in shell thickness were observed in the 10 % HFLM, Rayabold-only, and 15 % HFLM + Rayabold groups. The lack of a direct correlation between shell weight and shell thickness suggests potential changes in shell structure beyond simple increases in calcium deposition. This decoupling may indicate improvements in eggshell density, porosity, or crystalline micro-architecture—all factors that contribute to mechanical strength and reduced breakage (Ketta & Tůmová, 2016). Given that eggshell thickness is closely linked to shell strength and transport resilience, these enhancements may have considerable economic relevance for commercial egg producers (Ketta & Tůmová, 2016).

These findings are in agreement with prior studies that demonstrated improved shell quality with insect-derived ingredients and functional feed additives such as probiotics and enzymes (Liu et al., 2021; Xu et al., 2023; Huang et al., 2024; Wang et al., 2024; Zammit & Park, 2024). However, some earlier studies have reported negative effects on shell quality at higher insect meal inclusion levels, potentially due to mineral imbalances or reduced feed palatability leading to lower overall nutrient intake (Agunbiade et al., 2007). These contrasting results underscore the importance of carefully calibrating both HFLM inclusion levels and supplement strategies to maximize benefits while mitigating risks.

Additionally, Rayabold supplementation, both alone and in combination with 15 % HFLM, significantly increased yolk height relative to the control. Yolk height is an important indicator of yolk quality and freshness and is closely associated with nutrient density and lipid deposition. The improvement observed in this study suggests enhanced nutrient partitioning to the yolk, potentially driven by improved digestibility and absorption of lipids and fat-soluble vitamins. These effects are likely attributable to the combined action of enzymes facilitating nutrient release and probiotics promoting efficient metabolic assimilation (Belhadj Slimen et al., 2023; Huang et al., 2024). This result is further supported by evidence that probiotic supplementation can enhance lipid metabolism and improve yolk development via gut microbiota modulation and increased bile salt activity (Xu et al., 2023).

#### 4.3. Blood parameters

The inclusion of HFLM in laying hen diets demonstrated a notable hypoglycemic effect, particularly at 5 % and 10 % inclusion levels. This finding is consistent with recent studies in broilers, such as that of Sajjad et al., (2024), who observed significantly reduced blood glucose concentrations in birds fed a 12 % BSFLM diet. Several compositional attributes of insect meals may underlie this response. The relatively high lipid content of HFLM provides a sustained energy source, which may delay gastric emptying (Gentilcore et al., 2006) and consequently attenuate postprandial glycemic spikes. Additionally, insect meals are rich in branched-chain amino acids, particularly leucine, which is known to enhance insulin secretion and facilitate glucose uptake (Newsholme et al., 2006; Belhadj Slimen et al., 2023). Chitin, a

structural polysaccharide in insect exoskeletons, may further modulate glucose absorption by slowing carbohydrate digestion in the gastrointestinal tract (Tzeng et al., 2022).

Nevertheless, not all studies have observed consistent hypoglycemic effects. Marono et al., (2017), for example, reported no significant effect of defatted BSFLM on glucose concentrations in poultry. These discrepancies may reflect variations in insect species (e.g., *Musca domestica* vs. *Hermetia illucens*), processing techniques (e.g., defatting, drying temperature), inclusion levels, and physiological differences among bird breeds, ages, or production stages.

In terms of lipid metabolism, the study revealed that 10 % HFLM inclusion produced the lowest cholesterol concentrations, indicating a potentially optimal threshold for hypocholesterolemic effects. Furthermore, triglyceride concentrations were significantly reduced in birds fed diets containing 5 % and 10 % HFLM with Rayabold supplementation. These results point to a synergistic interaction between insect meal and enzyme–probiotic supplementation in modulating lipid profiles. Similar lipid-lowering effects have been reported by Akpodiete et al., (1998) with HFLM and more recently by Sajjad et al., (2024) with BSFLM inclusion in broiler diets. Marono et al., (2017) also documented reductions in serum cholesterol and triglycerides in laying hens fed BSFLM compared to soybean-based diets. The mechanism behind this hypolipidemic response is likely multifactorial. Chitin has been proposed to bind bile acids in the intestine due to its cationic nature, thereby reducing lipid emulsification and absorption (Hossain and Blair, 2007). Hossain and Blair (2007) demonstrated that chitin supplementation in broiler diets led to dose-dependent reductions in serum cholesterol and triglycerides, with optimal effects observed at 50 g/kg.

The hepatic enzyme profile presented a more nuanced picture. AST levels were significantly reduced in hens fed diets containing 10 % and 15 % HFLM, particularly when combined with Rayabold. As elevated AST typically reflects hepatocellular damage, this reduction suggests improved hepatic integrity or reduced inflammatory burden. Insect meals are known to contain antioxidant and anti-inflammatory peptides that may confer hepatoprotective effects (Belhadj Slimen et al., 2023), and the addition of probiotics and enzymes may amplify this effect. For example, Derakhshan et al., (2023) reported decreased liver enzyme activity in poultry supplemented with enzyme–probiotic blends.

However, ALT levels were significantly elevated in the 15 % HFLM + Rayabold group, raising concerns about potential hepatic stress. Unlike AST, ALT is more liver-specific and a rise in its activity is often a clearer indicator of hepatocellular injury (Oh et al., 2017). One plausible explanation is that the high nutrient density of the 15 % HFLM + Rayabold diet may have increased metabolic burden, particularly hepatic lipid processing or detoxification demands. Indeed, Hamani et al., (2022) found that birds fed high HFLM diets exhibited hepatomegaly and splenomegaly, consistent with increased hepatic workload. Similarly, a recent meta-analysis by Fikri et al., (2024) confirmed that high BSFLM inclusion in layers increased ALT levels, supporting the notion of dose-dependent hepatic stress associated with insect meal feeding.

Encouragingly, all HFLM inclusion levels resulted in reduced ALP activity compared to the control, which may reflect improved phosphorus utilization or altered bone turnover (Sharma et al., 2014). Given that ALP is associated with both bone metabolism and hepatic bile flow, its reduction could indicate enhanced mineral balance, especially considering the phosphorus-rich nature of HFLM (Sharma et al., 2014). Sharma et al., (2014) noted that high dietary phosphorus tends to lower ALP activity in laying hens. Nevertheless, the exact mechanism remains unclear, and further studies are warranted to disentangle hepatic versus skeletal contributions to ALP variation.

Comparisons with prior literature on liver enzyme responses to insect meals reveal inconsistent findings. For example, Elahi et al., (2020) observed decreased ALP and ALT activity in broilers fed 8 % maggot meal. In contrast, Marono et al., (2017) and Biasato et al., (2018) found no significant enzyme changes in birds fed insect meals. These inconsistencies likely reflect differences in insect species, processing, and

experimental conditions.

#### 4.4. Optimal HFLM levels: welfare and economic considerations

In this study, while no formal behavioral assessments were conducted, the absence of mortality, clinical signs of distress, or significant adverse changes in biochemical parameters up to 10 % HFLM inclusion suggests that bird welfare was not compromised. However, the increased ALT levels in the 15 % HFLM + Rayabold group point to potential hepatic stress, emphasizing the need to balance nutritional benefits with metabolic safety. Improved FCR at 10 % HFLM may also reflect enhanced nutrient utilization and lower physiological stress, further supporting the welfare benefits of moderate inclusion levels (Malemajta et al., 2024; Veldkamp et al., 2024).

From a practical and economic standpoint, 10 % HFLM inclusion appears to offer an optimal balance between performance, bird health, and cost-effectiveness. Although the 15 % inclusion level yielded slightly improved feed efficiency and EW, it also raised concerns about liver function and FI, potentially undermining its economic and ethical justification. Given the high cost of insect protein compared to conventional sources, marginal gains at higher inclusion rates may not offset increased health risks or feed expenses. Studies indicate that a 10 % inclusion level can reduce overall feed costs by 5–8 % while maintaining production performance without adverse health impacts (Waithaka et al., 2022; Suttibak, 2023). Moreover, the scalability of HFLM production supports long-term sustainability goals, as it is based on circular economy principles that convert organic waste into high-quality protein with a lower environmental footprint (Hussein et al., 2017). Thus, 10 % HFLM inclusion represents a nutritionally effective, welfare-conscious, and economically viable approach for future poultry systems.

#### 5. Conclusion and future perspective

This study demonstrated that dietary inclusion of HFLM, particularly at 10 % and 15 %, with or without Rayabold supplementation, significantly enhanced productive performance in laying hens by reducing FI and improving FCR, PER, and EER. Although overall EP and most egg quality traits were unaffected, specific combinations—particularly 15 % HFLM with Rayabold—resulted in improvements in eggshell weight, thickness, and yolk height. HFLM inclusion also positively influenced blood biochemical parameters, including reductions in glucose, cholesterol, triglycerides, AST, and ALP, suggesting metabolic benefits. However, the elevated ALT levels observed in the 15 % HFLM + Rayabold group may indicate hepatic strain at higher inclusion levels. These findings suggest that 10 % HFLM, with or without Rayabold, represents a nutritionally effective, metabolically safe, and environmentally sustainable alternative protein source for layer diets.

Nonetheless, the study's 10-week duration and its focus on a single genotype (Lohmann LSL-Lite) may limit the applicability of the findings across different breeds and production systems. Moreover, the long-term safety and health implications of insect-based diets remain to be fully explored. Future research should investigate the effects of HFLM inclusion across diverse poultry genotypes, age groups, and housing systems over extended periods. Additionally, comprehensive evaluations of economic feasibility, food safety, consumer acceptance, and regulatory considerations will be essential to support the broader adoption of insect-derived proteins in commercial poultry nutrition.

#### Ethical statement (animal studies)

This experiment received approval from Razi University's Animal Ethics Committee in Kermanshah, Iran, ensuring all procedures followed strict animal welfare guidelines (IR.RAZI.REC.1404). These protocols adhere to European Union standards for animal protection and feed legislation.

#### CRedit authorship contribution statement

**Abolfazl Salehizadeh:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Mehran Torki:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Maryam Darbemamieh:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Seyed Davood Sharifi:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization.

#### Declaration of competing interest

The authors declare no competing interests.

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#### Data availability

Data will be made available on request.

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