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The effect of different dietary manipulations on haematological properties in Japanese quail

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SUMMARY

Quantitative and qualitative dietary conditions are crucial for physiological functions. Blood haematology, the indicator of immune regulation, is critically affected by dietary conditions. Despite their importance, the effect of macro- and micronutrient manipulation remains unknown. We used feed restriction, energy or protein restriction, and supplementation of leucine, methionine, or both on top of restricted-feeding to study the effect on haematological properties in Japanese quail. Fifty-six birds of six weeks of age were distributed into seven treatments; control, 20% feed restriction, 20% energy restriction, 20% protein restriction, supplementing 20% leucine, 20% methionine, or both on top of restricted feeding. Haematological properties, including white blood cell count, number and percent of lymphocytes, mid-range (eosinophil + basophil), granulocytes, red blood cell count, haemoglobin levels, packed cell volume, mean corpuscular volume, and mean corpuscular haemoglobin were analysed. We found that quantitative feed restriction, energy restriction, and amino acid supplementations showed no significant effect compared to the control group. However, protein restriction reduced all indices, suggesting the importance of proteins in maintaining leukocyte and erythrocyte indices. The present study revealed that neither moderate calorie restriction nor moderate specific amino acid supplementation have an impact on blood haematology, while 20% protein restriction has a notable effect.

Keywords: *Energy restriction; haematology; leucine; methionine; protein restriction*

INTRODUCTION

Blood haematological indices are essential biomarkers in monitoring the physiological functions of organisms, including immune function and stresses (Odunitan-Wayas et al., 2018; Talebi et al., 2005). Haematological properties are significantly affected by quantitative and qualitative dietary conditions via altering metabolic activities, consequently affect the fitness of organisms (Etim et al., 2014). Quantitative feed intake can affect blood cell counts such as white blood cells (WBC) and red blood cells (RBC) count, levels of haemoglobin (HGB), and platelets (Maxwell et al., 1990a, b). Feed quality can also have a significant effect on haematological indices (Kuzmina et al., 2021; Ogbuewu et al., 2017).

Quantitative and qualitative feed restriction have been used as a management strategy in poultry production. Moderate feed restriction has been applied to slow down the rapid growth of specialised breeders and prevent excessive fat accumulation, thereby reducing metabolic disorders, increasing healthy reproductive lifespan (Carneiro et al., 2019; Hassan et al., 2003). However, severe and prolonged restriction can lead to malnutrition and stress, and affect the immunity and wellbeing of birds. The effect of feed restriction could be assessed through haematological properties (Damgaard et al., 2012; Junqueira et al., 2003).

However, we have limited knowledge of what will happen if we supplement certain essential amino acids under restricted feeding conditions. Under laboratory conditions, feed restriction and supplementation of amino acids could separate the effect of total energy intake and specific amino acids on haematological properties. Methionine and leucine are essential amino acids that play a crucial role in the growth and development of birds (Fagundes et al., 2020). Methionine is often considered the first limiting amino acid in poultry diets. Leucine is another essential amino acid required for protein synthesis and inhibits protein degradation (Garlick, 2005; Kratei and Shahir, 2021). Supplementing methionine, leucine, or both under feed restriction conditions could be crucial for understanding these amino acids' specific roles in blood haematology. It is also important to study the effect of the concurrent or separate supplementation of methionine and leucine. Supplementation of these essential amino acids could also result in a negative result due to an amino acid imbalance (Fisher and Shapiro, 1961; Kidd et al., 2021).

Additionally, macronutrients, such as carbohydrates, lipids, and proteins, have an indispensable role in regulating haematological properties. Carbohydrates, the function of metabolisable energy, could affect blood haematology. However, while studies have reported about the effect of total calorie restriction (Damgaard et al., 2012; Junqueira et al., 2003), the effect of metabolisable energy restriction under full feeding is not known. Furthermore, the protein content of a feed is essential for shaping haematological profile of animals, where

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protein deficiency can cause anaemia, the sigh of reduced red blood cell count, haemoglobin level (Ahmed and Maqbool, 2017; Etim et al., 2014). However, less information is available showing the regulatory role of protein restriction compared to full feeding and feed-restricted conditions.

We applied methionine and leucine supplementation on top of restricted feeding and protein and energy restriction to assess haematological parameters. We used Japanese quails (*Coturnix japonica*) as an experimental avian model. The Japanese quail is a well-known avian model for studying physiological and phenotypic fitness in response to treatments. The domesticated Japanese quail is retaining several experimentally suitable characteristics, such as a smaller body size (Ottinger, 2007). We hypothesise that supplementation of methionine, leucine, or both curtails the effect of feed restriction. Additionally, energy or protein restrictions could have a negative effect on the haematological properties.

MATERIALS AND METHODS

The experiment was conducted in accordance with the EU Directive "Legislation for the protection of animals used for scientific purposes" and approved by the Ethical Committee for Animal Use of the University of Debrecen, Hungary (Protocol No. 5/2021/DEMAB).

Experimental animals, housing and treatments

We incubated 450 Japanese quail eggs for this study purpose on April 15, 2023. After hatching, 340 newly hatched chicks were reared under grower feeding up to four weeks and breeder feeding up to five weeks. At the age of five weeks, we selected and placed 56 female birds with similar body weights into individual cages for feed intake assessment and acclimation to the experimental conditions. Birds stayed for seven days under the *ad libitum* breeder feeding condition. During this period, we carefully monitored each individual's feed intake, which was used to calculate the amount of feed given to each bird according to its respective treatment. At the age of 6 weeks, birds were randomly assigned to seven treatments, as described in *Table 1*. Therefore, each treatment group contains eight birds. To control for positional effects (e.g., slight differences in light intensity along the cage height levels), we block the setup into eight blocks with an equal number of birds from all treatments.

The quail basal feed was formulated using soybean, corn, pea, and wheat meal in the range of nutrient requirements of breeder quails (*Table 2*; NRC, 1994). L-methionine and L-leucine were purchased from Vital-Trend Kft., Debrecen, Hungary. We added 20% methionine, 20% leucine, or both 20% methionine and 20% leucine on top of the recommended level and thoroughly mixed each. We also controlled for branched-chain amino acid imbalance. The metabolisable energy and protein restriction treatments were formulated by manipulating dietary ingredients.

Table 1. **Treatment codes, their description, and the number of birds per treatments**

No.	Treatment code	Description	Number of birds
1	Control	Full fed (birds received equivalent to their daily feed intake plus 5%)	8
2	FR	20% feed restriction	8
3	FR + L	FR + 20% Leucine	8
4	FR + M	FR + 20% methionine	8
5	FR + ML	FR + 20% methionine + 20% leucine	8
6	ER	Full feeding with 20% metabolisable energy restriction	8
7	PR	Full feeding with 20% protein restriction	8

Table 2. **Ingredient composition and nutrient level of experimental feed**

Ingredients	Inclusion rate%
Corn	25
Soybean meal	24.86
Wheat	10.1
Wheat bran	10
Pea	13.02
Oil	9.07
Limestone	5.83
Monoculcium phosphate	0.96
L-Methionine	0.19
L-Leucine	0
L-Threonine	0.07
Braced chain amino acids 2:1:1	0.02
Salt	0.38
Premixture[a]	0.5
Nutrient contents	
ME, MJ/kg	**12.13**
Crude protein, %	**18**
Lysine, **%**	1.014
Methionine %	**0.45**
Threonine, **%**	0.74
Tryptophan, **%**	0.216
Leucine, %	**1.42**
Ileucine, **%**	0.77
Arginine, **%**	1.26
Leu/Ile, ratio	1.84
Calcium, **%**	2.5
Phosphorus, **%**	0.608
non phytate P, **%**	0.35
Sodium, **%**	0.15

[a] 1 kg premix provided: 1000000 NE vitamin A, 200 000 NE vitamin D_3, 4900 mg/kg vitamin E, 200 mg vitamin K_3, 150 mg vitamin B_1, 500 mg vitamin B_2, 1200 mg Ca-d-Pantothetane, 400 mg vitamin B_6, 2 mg vitamin B_{12}, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 300000 mg choline cloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co.

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The low-energy diet was achieved by reducing the levels of carbohydrates- and lipid-containing ingredients (corn, soybean meal, and oil) and increasing the levels of wheat and pea. The energy-restricted group received a feed with 9.7 MJ/kg metabolisable energy. The low-protein feed was achieved by reducing the amount of wheat and soybean meal and increasing the levels of corn and pea. Therefore, the protein content of the feed given to the protein-restricted group was 14.4% (14.4 g/kg of feed) of the total feed. The trial was conducted for 14 days, and every morning, immediately after lights on, we removed the feeders before birds started eating, measured the remaining feed, and replenished the feeders with the pre-determined amount and type of feed. Daily feed intake was monitored, and any increase in feed intake in the control birds was used to adjust the amount of feed given to the other treatment group.

Blood sampling

At the 14 days of the experiment, all birds were euthanised by veterinary experts. A whole blood sample was collected from the jugular vein of each bird into a 5 mL EDTA tube to prevent clotting and properly labelled. A 20-µL blood sample was immediately added to buffer, and sent to the laboratory for haematological analysis on the same day.

Blood haematological analyses

Blood haematological properties were analysed using the URIT-3000 Vet Plus automated haematology analyser according to the manufacturer's operation manual (URIT-3000 Vet Plus, Orvostechnika Ltd., Budapest). A total of 20 µL of blood sample was added to 1 mL of dilution buffer and analysed for white blood cell count (WBC,10^9/L), red blood cells (RBC, 10^{12}/L), haemoglobin (HGB, g/dL), percent of lymphocyte (LYM%), lymphocyte count (LYM#), percentage (MID%) and number of (MID#) mid-range (eosinophil + basophil), percentage (GRAN%) and number of granulocyte (GRAN#), haematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg), and mean corpuscular haemoglobin concentration (MCHC, g/dL). Measurements were applied on the same day after sample collection. After measurements reference intervals of the haematological indices were calculated using Box-Cox transformation with the robust method in Microsoft Excel 2013 using RVA V2.1 (Geffré et al., 2011). The reference intervals are presented in *Table 3*. We cannot find accepted standard reference values for Japanese quails.

Table 3. **Reference intervals of the haematological properties in Japanese quails at 8 weeks of age**

Variables	Reference interval	90% CI for lower limit	90% CI for upper limit
WBC	69.42–183.40	63.39–75.26	174.74–191.97
LYM#	27.10–50.93	24.89–28.99	49.41–52.47
LYM%	24.35–64.14	20.81–28.09	61.17–67.07
MID#	10.71–14.81	9.61–11.45	14.67–14.94
MID%	9.77–23.16	8.97–10.64	22.08–24.76
GRAN#	34.92–62.19	33.28–36.53	59.82–64.70
GRAN%	25.21–95.35	22.40–28.28	89.29–100.38
RBC	2.46–5.49	2.21–2.76	5.13–5.79
HGB	11.55–23.95	10.77–12.47	22.43–25.49
PCV	35.74–72.44	33.58–38.27	67.92–76.55
MCV	120.60–138.96	119.22–122.19	137.94–139.97
MCH	39.77–47.46	39.34–40.17	46.77–48.21
MCHC	31.31–35.15	31.02–31.58	34.89–35.44

Abbreviation: WBC, white blood cell count (10^9/L); LYM#, lymphocyte count; LYM%, percent of lymphocyte; MID#, number of mid-range (eosinophil + basophil); MID% percentage mid-range; GRAN #, number of granulocyte; GRAN%, granulocyte percentage; RBC, red blood cells (10^{12}/L); HGB, haemoglobin (g/dL); PCV, packed cell volume (%); MCV, mean corpuscular volume (fL); MCH, mean corpuscular haemoglobin (pg); MCHC, mean corpuscular haemoglobin concentration (g/dL)

Statistical analysis

All data were analysed using R version 4.3.2, 'Eye Holes' (https://www.r-project.org/). We used a linear mixed effects model to analyse the effect of treatments on the haematological parameters, treatment considered as a fixed effect, while experimental block and time of laboratory analysis considered as random factor. The lmer function from the lme4 package (Bates et al., 2015) was used to estimate model parameters for both fixed and random effects. Additionally, the lmerTest v. 3.1-3 package (Kuznetsova et al., 2017) was employed to compute p-values in the ANOVA and model summary tables. For mean comparison, the emmeans function adjusted for Tukey was used as a *post-hoc* test with a $p < 0.05$ significance level (Lenth et al., 2018). For a reasonable comparison, we divide the data into two categories: energy and protein restriction and amino acid supplementation; hence, we make statistical analyses accordingly. In the energy and protein restriction category, we compared the control, FR, ER, and PR groups, whereas the amino acid supplementation groups were compared to each other and further with the FR group.

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RESULTS AND DISCUSSION

Protein restriction negatively affect haematological indices

The statistical results of the effect of feed restriction and energy and protein restrictions are presented in *Table 4*. Feed restriction has no significant effect on any of the haematological indices compared to the control group. Protein restriction showed the lowest values in most, with significant differences in some indices. This suggests that birds could maintain blood property up to 20% quantitative feed restriction. Interestingly, protein restriction group showed significantly lower white blood cell count (WBC) compared to the energy restriction group (p = 0.011), and marginally lower value than the control group (p = 0.056), while the energy restriction group is not different from control group. A similar effect was observed on the other WBC component, lymphocyte count (LYM#) and percentage (LYM%), mid-range (eosinophil + basophil) count (MID#) and percentage (MID%), number (GRAN#) and percentage (GRAN%) of granulocyte. Protein restriction treatment showed marginally reduced lymphocyte count compared to the control (p = 0.095) and quantitative feed restriction (p = 0.098) groups, whereas percentage lymphocytes were higher in the protein-restricted group. Additionally, the mid-range was significantly lower in the protein-restricted group compared to the control (p = 0.045) and energy-restricted (p = 0.017) groups. The number and percentage of granulocytes were lowest in the protein-restricted group.

The lower WBC components in the protein-restricted groups suggested that protein content of a feed is more critical than energy content (Ahmed and Ahmad, 2020; Hastreiter et al., 2021; MacArthur et al., 2022). Dietary protein levels are crucial in maintaining the white blood levels and immune system in general. Proteins are sources of amino acids to be used by bone marrow for the production of leukocytes (Cunha et al., 2013; Hastreiter et al., 2021). Therefore, low dietary protein levels (protein restriction) could expose birds to a reduced supply of required amino acids and immune capacity compromised. A study on broiler chicken reported that a 15% lower protein reduced leukocyte levels (Jahanian, 2009). It also revealed that under adequate protein content, animals could maintain immune capacity under moderate metabolisable energy restriction. Moderate energy restriction could also improve the levels of leukocytes by reducing inflammation and oxidative stresses. This may improve the function of leukocytes and improve adaptive immune response. However, long-term severe energy restriction could impair the immune system and expose birds to disease.

Table 4. **Effect of energy and protein restriction on haematological properties**

Variables	Control	FR	ER	PR	Pooled SEM	p-value
WBC	130.60$^{a\bullet}$	122.68^{ab}	136.10^{a}	100.94$^{b\bullet}$	8.31	0.007
LYM#	49.50$^{\bullet}$	49.56$^{\bullet}$	47.29	43.18$^{\bullet}$	4.52	0.083
LYM%	38.72^{ab}	40.34^{ab}	35.94^{b}	43.67^{a}	1.72	0.017
MID#	17.18^{a}	16.56^{ab}	17.60^{a}	14.62^{b}	1.11	0.011
MID%	13.30	13.44	13.00	13.89	0.30	0.199
GRAN#	65.16^{a}	58.76^{ab}	70.29^{a}	43.58^{b}	5.73	0.005
GRAN%	48.80$^{ab\bullet}$	46.52^{ab}	51.04^{a}	42.99$^{b\bullet}$	1.91	0.015
RBC	3.94	3.92	4.17$^{\bullet}$	3.46$^{\bullet}$	0.19	0.078
HGB	16.80^{ab}	16.96^{ab}	18.56^{a}	14.84^{b}	0.88	0.021
PCV	51.20^{ab}	51.53^{ab}	55.66^{a}	44.75^{b}	2.67	0.027
MCV	132.42^{ab}	130.36^{ab}	134.17^{a}	128.29^{b}	1.84	0.042
MCH	43.60	42.83	44.63$^{\bullet}$	42.64$^{\bullet}$	0.73	0.057
MCHC	33.02	32.85	33.41	32.95	0.39	0.537

Data are represented by the mean from 8 birds per group. Means followed by a common letter in a raw are not significantly different at $p < 0.05$. Dots next to means or letters indicates marginaly insignificant difference ($p < 0.1$). Abbreviation: Control, full fed group; FR, 20% feed restriction; ER, 20% metabolisable energy restriction; PR, 20% energy restriction. Abbreviations of variable names from *Table 2*.

Our results also indicate that protein restriction resulted in lower erythrocyte indices (*Table 3*). Red blood cell count was marginally lower in the protein restriction group compared to the energy-restricted group (p = 0.05). Haemoglobin level (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) were significantly lower in the protein-restriction group than the energy restriction group (HGB: p = 0.016, PCV: p = 0.023, MCH: p = 0.020, MCH: p = 0.023). Dietary protein plays a critical role in the health and function of red blood indices (Orten and Orten, 1943; Sakthivel, 1988). Protein provides amino acids crucial for the production of haemoglobin and the cellular structure of red blood cells. Therefore, under lower dietary protein levels, the body cannot synthesis sufficient haemoglobin and red blood cells, which results in reduced levels of the other indices. The justification for the no effect of 20% metabolisable energy restriction on erythrocyte indices is similar to the justification for the effect on leukocytes.

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Amino acid supplementation have no effect on haematological properties

As it is mentioned above, the 20% feed restriction treatment did not affect any of the haematological properties compared to the control group. Therefore, in this section, we present the effects of supplementing leucine, methionine, or both, in addition to the recommended proportion, on the haematological properties of quantitatively feed-restricted groups. The output showed that none of the amino acid supplementation treatments resulted in any significant effect on all of the haematological indices (*Table 5*). We also compared them to the control group and none of them showed a significant difference. This is in contradiction with the previous findings that suggested that amino acid supplementation changed the haematological properties depending on the type and levels of supplementation. For example, Lugata et al. (2022) reported that either adding methionine or reducing 10% methionine from the recommended level reduces most of the indices in layer chicken. Another study on broilers reported that supplementation of 10%–50% methionine reduced the leukocyte indices, whereas only the 50% supplementation showed a reducing effect on erythrocyte indices (Carvalho et al., 2020). Another study also reported that supplementation of 10% to 20% methionine showed no effect on leukocyte indices (Nikoofard et al., 2016). We cannot corroborate our finding with studies from leucine supplementation.

Table 5. **Effect of amino acid supplementation on haematological properties**

Variables	FR	FR + L	FR + M	FR + ML	Pooled SEM	p-value
WBC	124.26	117.56	122.46	122.84	9.87	0.214
LYM#	50.7	49.15	44.34	49.93	5.48	0.683
LYM%	40.19	41.24	39.89	40.85	40.19	0.568
MID#	16.6	15.75	16.06	16.97	1.09	0.707
MID%	13.44	13.57	13.59	13.74	0.28	0.901
GRAN#	59.36	54.65	56.47	56.94	6.41	0.942
GRAN%	46.71	45.17	46.57	45.45	2.30	0.932
RBC	3.92	3.86	3.97	3.87	0.19	0.979
HGB	16.80	16.39	17.06	17.01	0.85	0.949
PCV	51.09	48.90	50.89	50.60	2.45	0.921
MCV	130.33	127.90	127.95	130.42	1.49	0.330
MCH	42.83	42.45	42.89	43.89	0.60	0.236
MCHC	32.88	33.22	33.46	33.68	0.31	0.261

Data are represented by the mean from 8 birds per group. Abbreviation: FR, 20% feed restriction; FR + L, 20% leucine supplementation on top of restricted feeding; FR + M, 20% methionine supplementation on top of restricted feeding, FR + ML, 20% leucine and methionine together on top of restricted feeding. Abbreviations of variable names from *Table 2*.

CONCLUSIONS

The present study revealed that moderate quantitative feed restriction and energy restriction (20%) have no noticeable effect on any of the haematological properties, suggesting the beneficial effect of moderate calorie restriction. However, protein restriction (20%) reduced the levels of most of the parameters, suggesting the importance of protein over energy in maintaining leukocyte and erythrocyte indices. Furthermore, dietary supplementation of essential amino acids, leucine and methionine, on top of restricted feeding has no observable effect on blood haematology, suggesting that either alone or together of these amino acids are not sufficient to impact haematological properties. Overall, this study revealed the comparative importance of quantitative and qualitative dietary manipulations on blood haematological properties in the less studied avian model, Japanese quail.

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