Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v40i1.38000

NONRUMINANT NUTRITION

Immune response, blood parameters and growth performance in broiler fed reduced protein diet supplemented with β-hydroxy-β-methyl butyrate and conjugated linoleic acid

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ABSTRACT. In this experiment a total of 300 d-old broilers (Ross 308) were randomized across 20 floor pens and reared up to 42 d. Treatments includes: 1) A normal-protein diet (NPD) was formulated according to the Ross 308 recommendation (2014) by corn and soybean meal, 2) A reduced-protein diet (RPD) was prepared with dietary protein reduced by 12 g kg^{-1} (1.2%) relative to the NPD, 3) HMB+ RDP, 4) CLA+RDP and 5) CLA+ HMB+ RDP. The results showed that broilers fed HMB diet had higher Average Daily Gain (ADG) and Feed Conversion Rate (FCR) significantly improved by HMB during the grower or the whole period ($p < 0.05$). Titer of serum antibodies of Newcastle disease was maximum in treatment supplemented with CLA+HMB compared to the control (RPD) in day 42. In comparison with the control, feeding CLA increased Lymphocyte and decreased heterophil and H/ L ratio ($p < 0.05$). Insulin hormone secretion increased by dietary HMB ($p < 0.05$). Red Blood Cells (RBC) and Hemoglobin (Hb) was significantly increased by HMB supplemented diet ($p < 0.05$). It could be concluded that supplementations of HMB and CLA together in reduced-protein diet have positive effect on growth performance, immune system and hematological parameter.

Keywords: hematological parameter; insulin; blood cell; low protein diet; protein synthesis.

Resposta imune, parâmetros sanguíneos e crescimento da performance dos frangos de corte alimentados com dieta de proteína reduzida e suplementada com beta-hidroxi-beta-metilbutírico e ácido linoleico conjugado

RESUMO. Um total de frangos com 300 dias de vida (Ross 308) foram aleatoriamente distribuídos em 20 recintos fechados e criados por 42 dias. Os tratamentos incluem 1) Dieta normal em proteínas (NPD) foi formulada de acordo com a recomendação para os Ross 308 (2014) a dieta consiste em milho e soja, 2). Uma dieta de proteínas reduzida (RPD) foi preparada com as proteínas reduzidas em 12 g kg^{-1} (1.2%) em comparação com o NPD, 3) HMB+ RDP, 4) CLA+RDP e 5) CLA+HMB+RDP. O resultado mostrou que os frangos alimentados pela dieta HMB tiveram uma maior Média de Ganho Diário (ADG) e Taxa de Conversão de Alimentos (FCR) significativamente melhorada pelo HMB durante o crescimento ou por todo o período ($p < 0.05$)., concentrações de soro de anticorpos da doença de Newcastle foi maximizada no tratamento suplementado com CLA+HMB comparadas com o controle (RPD) no dia 42. Na comparação com o controle, a alimentação CLA aumentou o Linfócitos (L) e diminuiu o heterofilo (H) e a proporção H/L ($p < 0.05$). A secreção do hormônio da insulina foi aumentado pela dieta HMB ($p <0.05$). Células Vermelhas sanguíneas (RBC) e a hemoglobina (Hb) foi significantemente aumentadas pelo HMB ($p < 0.05$). E pode-se concluir que as suplementações de HBM e CLA, juntas na dieta de proteína reduzida, tem um efeito positivo no crescimento, sistema imunológico e também no parâmetro hematológico.

Palavras-chave: parâmetro hematológico; insulina; célula sanguínea; dieta baixa em proteínas; síntese proteíca.

Introduction

Efficient use of nutrients in order to maximize production of poultry meat is one of the most important goals of poultry nutrition science (Khoramabadi, Akbari, Khajali, Noorani, & Rahmatnejad, 2014). Supplementation should avoid the excess of nutrients in the formulated diets and consequently, the unnecessary expenses with nutrition. The reduction of crude protein (CP) in diets, properly supplemented with crystalline amino acid, could help to reduce nitrogen excretion in feces, urine and litter quality (Khajali & Wideman, 2010). It is also reducing the energy expenditure associated with the excretion of excess of dietary CP as uric acid and lowers the metabolic heat production in comparison to higher levels of CP in diet. However, this energy should be used for production (Le Bellego & Noblet, 2002). The nutritional principle involved is to use of dietary supplements such as β-Hydroxy-β-methyl Butyrate (HMB) and Conjugated Linoleic Acid(CLA) that protein and energy may be used with high efficiency and direct it towards growth performance.

On the other hand, the humoral and cellular immune systems of chicken were affected by protein deficiency. Moreover, protein deficiency negatively affected the cellular immunity by decreasing lymphocyte number and overall white blood cell numbers and increase in the Heterophil/Lymphocyte ratio (H/L ratio) which is a stress indicator (Hindi, Elagib, & Elzubeir, 2012). The β-hydroxy-β-methylbutyrateis a natural, biologically active compound available in many dietary components such as catfish, alfalfa, asparagus, avocado, cauliflower and grapefruit (Qiao et al., 2013). Also, HMB is a metabolite of the essential branched-chain amino acid leucine, which product endogenously in small amounts and is one of the latest dietary supplements for the promotion of gains and lean body mass (Qiao et al., 2013). The β-hydroxy-β-methylbutyrate increases protein synthesis directly by activating the mammalian target of rapamycin (mTOR), the intracellular protein that controls protein synthesis.

Furthermore, dietary HMB supplementation seems to have enhancing properties on immune function in poultry (Peterson, Qureshi, Ferket, & Fuller Junior, 1999). Studies have shown that HMB have positive effects on proliferation of chicken macrophage cells and enhanced immune function. Flummer, Kristensen, and Theil (2012) found heavier spleens in piglets born from HMB-supplemented sows. He concluded that this may indicate an HMB mediated improvement in the immune function.

Conjugated linoleic acid (CLA), which are a mixture of positional (9, 11 or 10, 12) and geometric (cis and trans) isomers of linoleic acid have been ascribed several biological activities (Simon, Männer, Schäfer, Sagredos, & Eder, 2000). There is no consistency in previous reports concerning the effects of CLA on the chicken's weight gain. Thiel-Cooper, Parrish Junior, Sparks, Wiegand, and Ewan (2001) found a linear increase of daily weight gain associated with CLA supplementation. There are also reports on the moderate weight loss caused by the dietary CLA in chickens. Nevertheless, a lower daily weight gain was observed in CLA fed chicks (Suksombat, Boonmee, & Lounglawan, 2007).These changes in metabolism reflected in blood serum levels of intermediary metabolites.

The objective was therefore to evaluate HMB and CLA supplementation in reduced protein diet on grows performance, immune system and hematological index in broiler chickens.

Material and method

Animal and treatments

Total of 300 day-old broilers (Ross 308) were randomized across 20 litter pens. Day-old broiler chickens were raised on a commercial diet until seven days of age. Seven-day-old chickens were then allocated to pens (15 birds per pen) so that all pens had similar initial body weights. Dietary treatments were formulated (Table 1) for the growing (7 to 24 days of age) and finisher (24 to 42 days of age) these stages were according to the Ross 308 recommendations. A commercial broiler diet with normal-protein content (NDP) was prepared as control (treatment 1). A reduced-protein diet (RPD) was prepared with 12 g kg^{-1} (1.2%) lower crude protein (treatment 2). The RPD was also supplemented with HMB, CLA and HMB + CLA as treatments 3, 4 and 5 respectively. The HMB (KAREN Pharma and Food Supplement Co) used in this study were directly added to the complete diet (3g kg^{-1}). The CLA (Muscle Pharm) supplement was 0.5% used in this study, contained 10% isomer 9c, 11t and 10% isomer 10t, 12c of conjugated linoleic acid. Feed were offered *ad libitum* in all period of experiment. Light was provided 24h a day and was gradually reduced until 23h a day. The temperature was also gradually reduced by 2°C per week from the initial 32°C. Weight gain and feed intake were recorded weekly. The average daily gain (ADG), feed conversion rate (FCR) and feed intake (g d^{-1}) were calculated in this respect.

Serum titers of Newcastle disease (ND) and Sheep Red Blood Cell (SRBC)

In order to measure the antibody titer against Newcastle disease as immune responses, at 27 and 42 days of age blood samples were collected from wing vein through non-heparinized tubes for determining the serum titers of Newcastle disease.

Whole sheep blood collected in heparinized tube was washed three times in phosphate buffered saline (PBS, pH7.4) and diluted in PBS to 25 per cent (v v^{-1}). The chicks were immunized with 1 mL of 25 per cent SRBC (Kundu, 1999) in breast muscles at day 28. Booster dose of SRBC antigen was given at 35 day. Blood samples were collected at days 35 and 42 of age for assessing hemaglutination (HA) titre (Abdel-Ati, Latshaw, & Donahoe, 1984) against SRBC by using freshly prepared one per cent SRBC.

Table 1. Composition and calculated of the experimental diets analysis.

Ingredients	Period of examine				
	0-7	8-24		25-42	
	NPD[1]	NPD	RPD[2]	NPD	RPD
Corn	52.2	50.64	55.04	55.73	60.07
Soy bean meal	40.85	39.74	35.87	34.34	30.53
Soy bean Oil	2.45	5.73	4.93	6.30	5.52
Dicalcium phosphate	1.92	1.68	1.72	1.51	1.55
Limestone	1.05	0.96	0.97	0.89	0.9
Vitamin[3] and Mineral[4] premix	0.5	0.5	0.5	0.5	0.5
DL-Methionine	0.37	0.3	0.34	0.27	0.31
L-Lysine	0.19	0.04	0.17	0.05	0.19
NaCl	0.3	0.3	0.3	0.3	0.3
$NaHCO_3$	0.1	0.1	0.1	0.1	0.1
L- Threonine	0.07	0.01	0.06	-	0.05
Total	100	100	100	100	100
Calculated values					
ME (kcal kg^{-1})	2900	3100	3100	3200	3200
Crude protein (%)	22.33	21.5	20.31	19.5	18.33
Calcium (%)	0.96	0.87	0.87	0.79	0.79
Available phosphorus (%)	0.48	0.43	0.43	0.39	0.39
Sodium (%)	0.16	0.16	0.16	0.16	0.16
Lysine (%)	1.44	1.29	1.29	1.16	1.16
Methionine (%)	0.7	0.63	0.64	0.57	0.59
Methionine + Cystine(%)	1.08	0.99	0.99	0.91	0.91
Threonine (%)	0.97	0.88	0.88	0.79	0.78
Tryptophan (%)	0.28	0.27	0.25	0.24	0.22

[1]Normal protein diet, [2]RPD: Reduced Protein Diet. [3]Vitamin premix supplied the following per Kg of diet: vitamin A (retinol), 8400 IU; vitamin D3 (cholecalciferol), 1800 IU; vitamin E (tocopheryl acetate), 150 mg; vitamin K, 24 mg; B1, 8 mg; B2, 16.6 mg; B6, 13 mg; B12, 5 mg; panthothenic acid, 12 mg; niacin, 36 mg; biotin, 10 mg; folic acid, 2.2 mg; choline chloride, 128.8 mg; antioxidant, 100 mg; [4]Mineral premix supplied the following per Kg of diet: Fe (FeSO4, 20.1% Fe), 95 mg; Mn (MnSO4, 32.5% Mn), 120 mg; Zn (ZnO, 80.5% Zn), 120 mg; Cu (CuSO4, 30.3% Cu), 35 mg; I (KI, 58% I), 5 mg; and Se (NaSeO3, 45.5% Se), 2.2 mg.

Blood hormone measurement

Serum Growth Hormone (GH), and insulin concentrations were analyzed using commercially available Pars Azmon kits (Pars Azmon, Tehran, Iran).

Hematological analysis

Hematological analysis was carried out using the blood collected from the experimental chickens at the end 21 days of age. The birds were starved for 8 hours and blood samples were collected from the wing vein of two selected birds per treatment. Twelve milliliter of blood was collected from each chicken and transferred immediately into test tubes with EDTA as anticoagulant for the direct measurements of hemoglobin (Hb), red blood cells (RBC), white blood hematology. Cells (WBC), Hematocrit Test (HcT), Mean Corpuscular Volume (MCV) and Placet Test (PcT) were calculated. Blood smears were also performed to count lymphocytes (L), heterophils (H) and monocyte, which made it possible to determine the H: L ratio.

Statistical analysis

All data were analyzed as a completely randomized design according to a 2×2+1 factorial, using the GLM procedure of Statistical Analysis System (SAS, 2004), and means were compared by Tukey's test at the 5% probability level.

Result and discussion

The effect of HMB and CLA supplementation of RPDs on growth performance of broiler chickens is shown in Table 2. Supplementation of HMB (0.3%), the highest level ever used in broiler, with CLA would favorably effects on the growth performance. The ADG was increased in the finisher stage and throughout the feeding trial with HMB compared with the negative control (RPD) ($p < 0.05$). Feed intake also increased significantly with supplementation of HMB in RPD in the entire period ($p < 0.05$). However, the FCR in treatments contained HMB significantly improved compared with the negative control (RPD). Dietary CLA supplementation does not have significantly effect on growth performance of broiler.

Table 2. Effect of treatments on growth performance in broiler chickens reared up to 42 day of age (g).

	7-21day			21-42 day			7-42 day		
Treatment	FI[4](gd^{-1})	ADG[5](g)	FCR[6]	FI(gd^{-1})	ADG(g)	FCR	FI(gd^{-1})	ADG(g)	FCR
NPD[1]	40.4	27.1	1.49	175^{b}	92.3^{b}	1.89^{ab}	118^{b}	65.1^{b}	1.82^{ab}
RPD[2]	39.6	27	1.47	177^{ab}	91.1^{b}	1.95^{a}	119^{b}	64.4^{b}	1.85^{a}
RPD+ CLA[3]	40.5	28.7	1.40	179^{ab}	96.5^{b}	1.86^{ab}	121^{ab}	68.2^{b}	1.78^{ab}
RPD+HMB[4]	40.6	28.3	1.43	193^{a}	108.1^{a}	1.79^{b}	130^{a}	74.8^{a}	1.73^{b}
RPD+CLA+HMB	40.5	28.4	1.42	183^{ab}	100.2^{ab}	1.83^{b}	124^{ab}	70.3^{ab}	1.76^{ab}
SEM	0.42	0.57	0.02	3.75	2.2	0.02	2.27	1.42	0.02
P-Value									
CLA	0.33	0.11	0.18	0.26	0.58	0.41	0.41	0.82	0.29
HMB	0.25	0.41	0.71	0.016	0.0003	0.01	0.01	0.0005	0.007
CLA*HMB	0.28	0.17	0.29	0.14	0.008	0.09	0.09	0.01	0.03

NPD: Normal Protein Diet, RPD: Reduced Protein Diet, CLA: Conjugated Linoleic Acid, HMB: β-Hydroxy-β-methyl Butyrate, [4]ADG: Average Daily Gain, [5]FI: Feed Intake and [6]FCR: Feed conversation Rate. [a, b, c] Mean values within the same row sharing a common superscript letter are not statistically different at $p < 0.05$.

Similar result was reported by Qiao et al. (2013), in which chickens fed HMB supplemented diet were significantly heavier. However, some studies showed that different dietary level HMB supplementation did not significantly affect broiler weight gain (Peterson et al., 1999). Studies have shown that administration of HMB into the amnion of late-term avian embryo beneficially affected chick body weight (Uni, Ferket, Tako, & Kedar, 2005). Increasing in feed intake by HMB supplemented could be related to more body weight that need to more feed consumption to supply their requirements. However, Qiao et al. (2013) and

Buyse et al. (2009) have been reported no significant change in feed intake in broiler fed diet supplemented with HMB, this difference in results may be related to higher level of HMB supplemented in this study.

The HMB supplementation improved FCR, so Buyse et al. (2009) shown decreased FCR by dietary HMB supplementation. Nissen et al. (1996) explained that the increase in performance of broilers fed a diet supplemented with 0.003 to 0.01% HMB was possibly due to meeting the HMB requirement for metabolic function in these chicks. Rapid growth rate and the stress-surrounded environment may increase the HMB requirement.

The mean titer of serum antibodies of Newcastle disease was maximum in treatment supplemented with CLA+HMB compared to the control (RPD) in day 42 ($p < 0.05$), But there was no significant different at day 28. However, there was no significant difference in the antibody titer against SRBC among treatment groups on day 7 after primary immunization, and booster injection (Table 3).

Table 3. Effect of treatments on Serum titers of Newcastle disease and SRBC in broiler chickens at 28, 35 and 42 days of age.

	28 day	42 day	35 day	42 day
Treatment	ND[5]	ND	SRBC	SRBC
NPD[1]	2.5	1.00[b]	2.00	4.25
RPD[2]	2.75	1.25[b]	2.00	4.75
RPD+ CLA[3]	2.75	1.75[ab]	1.75	4.75
RPD+HMB[4]	2.75	2.00[ab]	2.50	5.5
RPD+CLA+HMB	3	2.75[a]	2.50	6.75
SEM	0.231	0.322	0.423	0.93
P-Value				
CLA	0.599	0.071	0.771	0.512
HMB	0.599	0.016	0.160	0.160
CLA*HMB	0.599	0.704	0.771	0.512

[1]NPD: Normal Protein Diet, [2]RPD: Reduced Protein Diet, [3]CLA: Conjugated Linoleic Acid, [4]HMB: β-Hydroxy-β-methyl Butyrate and [5]ND: Newcastle disease[a, b, c] Mean values within the same row sharing a common superscript letter are not statistically different at $p < 0.05$.

In vivo studies revealed HMB supplementation's positive influence on primary and secondary humoral response after injecting sheep's erythrocytes suspension (Peterson et al., 1999). In the study by Long et al. (2011), the immune regulatory actions of CLA of relevance to viral disease pathogenesis and immune responses were investigated. Their results indicated that dietary CLA enhanced immune function in chickens, particularly those of the IBDV (Infectious Bursal Disease Virus) immunosuppressive status.

Another striking beneficial effect of HM Band CLA observed in the current study was a significant increase in WBC and lymphocytes by CLA+HMB and CLA respectively ($p < 0.05$) and a decrease in Hetrophel and H: L rate at d21 in CLA supplemented diet ($p < 0.05$) with compared to control (Table 4), which showed an improvement in immune system. Also, increasing in lymphocyte populations may be indicative of higher activity of humeral immune responses in chicks fed HMB and CLA supplemented diets. Long et al. (2012) investigated the immune regulatory actions of CLA and suggested that CLA alleviated the immunosuppression of T lymphocytes in broiler chickens exposed to cyclosporine A through increased peripheral blood T lymphocyte proliferation and interleukin-2 levels. The elevated lymphocytes as CLA inclusion level increases could be a physiological adjustment against negative antigenic effects associated with the diet. In over all, our results have shown that immune stimulatory effects of HMB and CLA could help each other and significantly improve WBC and Newcastle disease antibodies in broiler chicken blood sample.

Table 4. Effect of treatments on leukocyte count and white blood cell in broiler chicken at 21 days of age.

Treatment	WBC[5] Num mm^{-3}	L[6] (%)	H[7] (%)	H/L	M[8] (%)
NPD[1]	12989[b]	36.5[b]	46.7[a]	1.28[a]	14.5
RPD[2]	12961[b]	36.5[b]	46.5[a]	1.27[a]	14.2
RPD+ CLA[3]	15352[ab]	42.2[a]	40.7[b]	0.97[b]	15
RPD+HMB[4]	15512[a]	41.2[ab]	43.5[ab]	1.05[ab]	15.5
RPD+CLA+HMB	15602[b]	41.0[ab]	42.2[ab]	1.03[b]	15.2
SEM	575	1.15	1.14	0.053	0.397
CLA	0.047	0.03	0.007	0.008	0.539
HMB	0.027	0.149	0.52	0.164	0.079
CLA*HMB	0.064	0.019	0.067	0.022	0.228

[1]NPD: Normal Protein Diet, [2]RPD: Reduced Protein Diet, [3]CLA: Conjugated Linoleic Acid, [4]HMB: β-Hydroxy-β-methyl Butyrate.[5]WBC: White Blood Cells, [6]L: lymphocytes, [7]H: Heterophils and [8]M: Monocyte[a, b, c] Mean values within the same row sharing a common superscript letter are not statistically different at $p < 0.05$.

The effect of treatments on blood hormone at 42 d of age is presented in Table 5. The blood insulin hormone significantly increased in treatment supplemented with HMB ($p < 0.05$). As noted previously, leucine and their metabolites such as HMB regulating the release of endocrine hormones (such as insulin and growth hormone) that is essentially important for the regulation of protein metabolism. Insulin is a peptide hormone that can stimulate muscle protein synthesis at the initiation level (Pause et al., 1994). Leucine is shown to be an insulin secretagogue that induces glucose-stimulated insulin secretion in pancreatic cells (Tsuruzoe et al., 1998). Another hormone we tested is GH. There was no significant change in GH level. Tatara (2008) observed that oral administration of HMB in neonatal lambs increased GH and IGF-1 concentration by 70% at 3 wk of life, whereas these hormones reached similar values to those in controls at 130 d of age. However, Qiao et al. (2013) did not show any significant change in GH levels following HMB supplementation in broiler chickens.

Table 5. Effect of treatments on blood hormone in broiler chickens at 42 days of age.

Treatment	Insulin	GH
NPD[1]	5.87[ab]	0.67
RPD[2]	5.7[b]	0.67
RPD+ CLA[3]	5.95[ab]	0.68
RPD+HMB[4]	6.12[a]	0.66
RPD+CLA+HMB	6.1[a]	6.75
SEM	0.085	0.013
p-Value		
CLA	0.13	0.19
HMB	0.005	0.75
CLA*HMB	0.25	0.79

[1]NPD: Normal Protein Diet, [2]RPD: Reduced Protein Diet, [3]CLA: Conjugated Linoleic Acid, [4]HMB: β-Hydroxy-β-methyl Butyrate, GH: Growth Hormone.[a, b, c] Mean values within the same row sharing a common superscript letter are not statistically different at $p < 0.05$.

The hematological parameters (Table 6) showed ($p < 0.05$) difference in all the blood components at different treatments except in monocytes, Hct and Plt. The red blood cells (RBC), hemoglobin (Hb) and mean corpuscular volume (MCV) were highly significant ($p < 0.05$) and increased in the diet contained HMB and HMB + CLA in compare to RPD (Table 5).

Table 6. Effect of treatments on Hematological analysis in broiler chickens at 21 and 42 days of age.

Treatment	RBC[5] 10^6 mm^{-3}	Hb[6] (g dL^{-1})	Hct[7] (%)	MCV[8] F L	PLt[9] Num mm^{-3}
NPD[1]	1.78[ab]	10.82[ab]	21.82	115.9[ab]	12.5
RPD[2]	1.55[b]	8.37[b]	18.65	115[b]	13
RPD+ CLA[3]	2.29[ab]	13.65[ab]	26.57	116.5[ab]	12.2
RPD+HMB[4]	2.52[a]	14.6[a]	28.8	115.4[b]	13
RPD+CLA+HMB	2.30[ab]	13.82[a]	27.55	118.4[a]	12.2
SEM	0.201	1.209	2.83	0.6	0.376
CLA	0.214	0.082	0.258	0.001	0.065
HMB	0.027	0.018	0.069	0.073	1.00
CLA*HMB	0.031	0.024	0.127	0.226	1.00

[1]NPD: Normal Protein Diet, [2]RPD: Reduced Protein Diet, [3]CLA: Conjugated Linoleic Acid and [4]HMB: β-Hydroxy-β-methyl Butyrate. [5]RBC: Red blood cells, [6]Hb: Haemoglobin, [7]HcT: Hematocrit Test, [8]MCV: Mean Corpuscular Volume and [9]PcT:Placet Test [a, b, c] Mean values within the same row sharing a common superscript letter are not statistically different at $p < 0.05$.

It has been observed by Esonu, Emenalom, and Udedibie (2001) that hematological constituents reflect the responsiveness of the animal to its internal and external environments which include feed and feeding. Eggum (1976) reported that hemoglobin (Hb) and packed cell volume (PCV) are very sensitive to the level of protein intake by poultry. In our experiment, RDP significantly decreased hemoglobin (Hb), red blood cells (RBC) and packed cell volume (PCV), however, dietary HMB or CLA improved these hematological parameters. It could indicated the effect of HMB on increases protein synthesis directly by activating mTOR, the intracellular protein that controls protein synthesis, which corresponded to the favorable conditions of hematological index. Significantly higher values of red blood cells, Hb and PCV were recorded in broilers fed high protein diets than those fed low protein (Mirtuka & Rawnsley, 1997). Talebi, Asri-Rezaei, Rozeh-Chai, and Sahraei (2005) have showed that the number erythrocytes of animals in good health vary with diets and clinical conditions of the animal.

Conclusion

That HMB increases growth performance and insulin secretion. Also, supplementations of HMB and CLA together in reduced-protein diet have positive effect on immune system and hematological parameter.

Acknowledgements

The authors would like to thank Bu-Ali Sina University for financial support and excellent scientific collaboration in department of Animal Science in Bu- Ali Sina University.

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Received on July 06, 2017.
Accepted on August 29, 2017.