ALIMET® (liquid methionine hydroxy analogue) in broiler chicken diets: immunity system, microflora population, and performance

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ADDITIONAL KEYWORDS

Broiler chicken.
Intestinal pH.
Serum antibodies.

SUMMARY

This experiment was conducted to investigate the effect of feeding increasing levels of ALIMET® (liquid methionine hydroxy analogue) in broiler chicken diets on serum antibodies, microflora population, and growth performance. The trial was designed as completely randomized design. A total of 300 unsexed 1-d-old broiler chicken (Ross 308) was randomly allocated to 4 treatments with 3 replicate of 25 chickens in each. Treatments were including of control and three graded levels of ALIMET® supplementation (i.e. 1.150, 1.437 and 1.725 g/kg in the starter and 0.450, 0.562, and 0.675 g/kg in the grower diets, respectively). As a result of this study, the weight gain increased linearly with increasing levels of ALIMET® in diets during 22 to 42 ($p \leq 0.001$) and 1 to 42 ($p \leq 0.017$) periods. There were no significant differences in the pH of the diets, ileum content, and excreta on days 21 and 42. A linear decrease ($p = 0.001$) in total number of bacteria was observed with increasing ALIMET® supplementation levels in the diets on 21 days of age. The antibodies titer of Newcastle Disease, Avian Influenza, and Infectious Bronchitis increased linearly with increasing ALIMET® supplementation levels in the diets on 21 days of age. Similar trend was found in antibody titer of Infectious Bronchitis on 42 days of age ($p = 0.006$). It concluded that the inclusion of ALIMET in broiler chicken diets have a beneficial effect on immunity and weight gain. However, effect of ALIMET® on diminution of harmful bacteria is not likely associated with pH reduction and is open to debate.

ALIMET® (análogo líquido de metionina hidroxilada) en el sistema inmunitario, población de microflora y rendimiento de broilers

PALABRAS CLAVE ADICIONALES

Pollo de engorde.
pH intestinal.
Anticuerpos séricos.

RESUMEN

Este experimento se realizó para investigar el efecto de niveles crecientes de ALIMET® (hidroxi análogo líquido de la metionina) en la dieta de broilers sobre los anticuerpos séricos, población microbiana y crecimiento. Se utilizó un diseño completamente al azar. Trescientos pollos Ross 308, no sexados fueron aleatoriamente asignados a 4 tratamientos con tres repeticiones de 25 pollos cada una. Los tratamientos incluían un control y tres niveles de suplementación de ALIMET® (1,150, 1,437 y 1,725 g/kg en las dietas de iniciación y de 0,450; 0,562, y 0,675 g/kg en las dietas de crecimiento, respectivamente). Como resultado, la ganancia de peso aumentó linealmente con el incremento de la proporción de ALIMET® en los periodos de 22 a 42 días ($p<0,001$) y 1 a 42 días ($p \leq 0,017$). No hubo diferencias significativas en el pH de las dietas, contenido ileal y excretas a los 21 o 42 días. Se observó un descenso lineal ($p= 0,001$) en el número total de bacterias al aumentar los niveles de ALIMET® en las dietas de 21 días de edad. El título de anticuerpos para enfermedad de Newcastle, gripe aviar y bronquitis infecciosa aumentó linealmente con el incremento de ALIMET® en las dietas de 21 días. Una respuesta similar se apreció para los anticuerpos de bronquitis infecciosa a los 42 días ($p<0,006$). Se concluye que la inclusión de ALIMET® en las dietas de broilers tiene un efecto beneficioso sobre la inmunidad y ganancia de peso. No obstante el efecto de ALIMET® sobre la disminución de bacterias patógenas probablemente no está asociado al descenso del pH y queda sujeto al debate.

INFORMACIÓN

Cronología del artículo.
Recibido/Received: 20.8.2014
Aceptado/Accepted: 17.11.2014
On-line: 16.3.2015
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INTRODUCTION

Sulfur amino acids are considered the first limiting in chicken corn-soybean meal diets (Leeson and Summers, 2000), and methionine (Met) is the first limiting ones in such diets. For many years, synthetic Met sources have been commonly supplied as either DL-Met or DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMB) which latter is added in a liquid free acid form or in a dry form as calcium salt. ALIMET®, 88 % aqueous solution of DL-HMB (Martin-Venegas *et al*., 2006), is a commercial Met source which used in broiler chicken diets (Saki *et al*., 2011). It is not an amino acid but rather an organic acid in that it bears a hydroxyl group on α-carbon instead of the amino group found in Met (Yi *et al*., 2007). Both Met sources are considered

Table I. Diets formulation and composition (g/kg) at starter and grower phase (Formulación de dietas y composición (g/kg) en la fase de inicio y crecimiento).

	Starter (1 to 21 day of age)				Grower (22 to 42 day of age)			
Items	T1	T2	T3	T4	T1	T2	T3	T4
Ingredients								
Corn grain	470	470	462	465	570	570	565	565
Soybean meal (48 % CP)	381	381	383	383	291	291	293.3	293.3
Wheat	30	30	30	30	35	35	32	32
Corn gluten meal (62 % CP)	50	50	50	50	60	60	60	60
Soybean oil	31	31	31	31	10	10	11	11.5
Dicalcium phosphate	16	16	16	16	13	13	13	13
Limestone	13	13	13	13	12	12	12	12
Common salt	3	3	3	3	3	3	3	3
Mineral-vitamin premix[1]	5	5	5	5	5	5	5	5
DL-methionine, 980 g/kg	1	—	—	—	0.4	—	—	—
ALIMET®	—	1.150	2.587	2.875	—	0.450	1.012	1.125
Total	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000
Calculated composition								
ME, kcal/kg	3 000	3 000	3 000	3 000	3 000	3 000	3 000	3 000
Crude protein (%)	217	217	217	217	190	190	190	190
Ca (%)	9.4	9.4	9.4	9.4	8.4	8.4	8.4	8.4
Available P (%)	4.2	4.2	4.2	4.2	3.3	3.3	3.3	3.3
Met (%)	4.7	4.7	4.7	4.7	3.6	3.6	3.6	3.6
Met + Cys (%)	8.4	8.4	8.4	8.4	6.7	6.7	6.7	6.7
Analyzed composition								
DM	869.3	868.3	862.9	865.7	869.5	869.1	869.2	865.2
CP	216.7	216.1	216.3	216.6	193.6	193.4	193.4	193.4
EE	54.5	54.5	54.2	54.3	36.8	36.7	38	38
CF	26.6	26.6	26.5	26.5	25.5	25.5	25.4	25.4

[1]Mineral premix: Mn, 80 mg; Zn, 84.5 mg; Fe, 80 mg; Cu, 5 mg; 1.1 mg; Co, 0.48 mg; Se, 0.30 mg/ kg of diet; vitamin premix: vitamin A, 11 000 IU (retinol); vitamin D_3, 3 000 IU; vitamin E, 50 mg (DL-α-tocopheryl acetate); vitamin k 3.5 mg; tiamin, 2 mg; riboflavin, 8 mg; calcium pantotenat, 12.4 mg; niacin, 50 mg; pyridoxine, 7 mg; pholic acid, 2 mg; vitamin B_{12}, 1.6 mg; biotin, 5 mg; choline chloride, 1.1 mg; antioxidant, 100 mg/ kg of diet. ME= Metabolisable energy.

equivalent sources of L-Met activity for hepatocyte protein synthesis and the differences have only been reported in mechanism and site of absorption (Dibner, 2003). They absorbed completely through gastrointestinal tract of poultry, converted to L-Met and utilized in protein synthesis (Richards *et al*., 2005).

Many studies have reported equivalent performance of broilers supplemented with DL-Met and DL-HMB on equimolar basis in the diets (Knight and Dibner, 1984; Garlich, 1985; Romer and Abel, 1999). However, there is conflict results in the last decade concerning the relative bioefficacy between DL-Met and DL-HMB in commercial broilers (Lemme *et al*., 2002; Daenner and Bessei, 2003; Dibner, 2003; Drew *et al*., 2003; Meirelles *et al*., 2003; Dibner *et al*., 2004; Motl *et al*., 2005; Liu *et al*., 2006; Vazquez-Anon *et al*., 2006a; Liu *et al*., 2007; Saki *et al*., 2011). Investigate of relative bioefficacy between synthetic Met sources and their effect on broiler performance is main focus of studies. Nonetheless, little information is available for broiler in relation to immune response and intestinal microflora population by Met sources, especially ALIMET®. Thus, the objective of the present experiment was to study effect of graded increasing levels of ALIMET® on immune response, microflora population, and performance of broiler chickens.

MATERIAL AND METHODS

The Animal Ethics Committee of the Agricultural Research Center of Qom-Iran approved the experiment (Nº 14-04-13-93030).

Experimental design and birds

A total of 300 unsexed 1-d-old broiler chickens (Ross 308) were weighted (initial body weights 48.33±2 g), sorted by weight and assigned randomly to 4 diets with 3 replicate of 25 broiler chickens in each. The corn-soybean meal diets **(table I)** were designed as starter (from 1 to 21 days) and grower (from 22 to 42 days) based on NRC (1994) recommendations. The trial was designed as completely randomized design. Treatments were including of control (Met addition at 1 g/kg in the starter and 0.4 g/kg in the grower diets, respectively) and 3 graded levels of ALIMET® (Novus International, Inc., Charles, MO, USA) supplementation (i.e. 1.150, 1.437, and 1.725 g/kg in the starter and 0.450, 0.562, and 0.675 g/kg in the grower diets, respectively). Broiler chickens were vaccinated with Newcastle Disease (ND), Avian Influenza (AI), and Infectious Bronchitis (IB) according to local veterinarian recommendations schedule (Salary *et al.,* 2014).

Broiler chickens were exposed to 23 h light and 1 h darkness. The temperature was maintained at 32 °C from 1 to 7 days of age, and then gradually reduced 3 °C per week until the temperature reached 25 °C. Feed (mash) and water were offered *ad libitum* for the entire duration of the experiment. Weight gain (WG) and feed intake (FI) were measured and feed conversion ratio (FCR) was calculated accordingly.

pH measurement

Two birds from each replicate were randomly selected and sacrificed by cervical dislocation on 21 and 42 days of age. The ileum content was genteelly removed and the pH was immediately measured. On day 21 and 42, 6 samples of excreta were randomly picked up from each replicate and the pH was immediately measured. The digesta diluted nine-fold (w/v) with distilled water were stirred for 5 min and the pH of the suspensions were measured using a calibrated pH meter (744, Metrohm, Herisau, Switzerland) (Kalantar *et al*., 2014). Six samples of feed from each replicate were gathered and the pH was measured. The pH of 0.1 % (w/v) feed suspensions were determined at 25 °C with a calibrated pH meter (744, Metrohm, Herisau, Switzerland) (Hemati Matin *et al*., 2013).

Microbial sampling and incubation

On days 21 and 42 of the experiment, 2 birds from each replicate were randomly selected and were slaughtered by cervical dislocation and immediately after dressing the complete intestinal tract was removed and transferred into an anaerobic chamber. The ileum contents were gently removed in sterile sampling tubes and immediately transferred on ice to the microbial laboratory. Serial dilutions of 1 g sample (10^{-4} to 10^{-7}) were made. Selective media of plate count agar, MacConkey agar, MRS agar plus 0.1 % of tween 80, and xylose lysine deoxycholate agar were inoculated to detect the total number of aerobic bacteria, coliforms, lactic acid bacteria, and *Salmonella*, respectively. Total number of aerobic bacteria and coliforms were counted after aerobic incubation for 24 h at 37 °C. The numbers of lactic acid bacteria were calculated after incubation in an anaerobic chamber at 37 °C for 48 h and *Salmonella* were counted after incubation for 24 h at 37 °C (Saki *et al*., 2010; Kalantar *et al*., 2014).

Immunological assay

Four birds from each replicate were randomly selected and blood samples were taken from wing vein on 21 and 42 days of age. Serum was separated by centrifugation of coagulated blood at 3000 r/min for 15 min. Antibodies titration against ND, AI, and IB diseases were performed using ELISA by specific kits of OVATEC® Plus, SERELISA® Rabies (Synbiotics, USA), and BIA-CK® 121 (Biochek, Netherlands), respectively (Salary *et al*., 2014).

Statistical analysis

All data were analyzed for normal distribution using the normal option of the univariate procedure of GLM procedure of SAS software (2008). A completely randomized design was employed. Data were analyzed by GLM procedure. Logarithmic (log10) transformation was applied for microbial colony forming unit (CFU). Polynomial orthogonal contrasts were carried out for ALIMET® levels to investigate the linear, quadratic, or cubic trends.

RESULTS

The growth performance of the broiler chickens is presented at **table II**. The results indicated that WG increased linearly with increasing ALIMET® levels during 22 to 42 ($p \leq 0.001$) and 1 to 42 ($p \leq 0.017$) days of age. No significant differences were observed in pH of the diets, ileum contents, and excreta on 21 and 42 days of age **(table III)**. A linear decrease ($p = 0.001$) in total number of aerobic bacteria was observed with increa-

Table II. Effect of the diets on weight gain (WG), feed intake (FI), and feed conversion ratio (FCR) of broiler chickens (Efecto de la dieta sobre la ganancia de peso (WG), consumo de alimento (FI) y la conversión alimenticia (FCR) de pollos de engorde).

	Treatments					p-value		
Item	T1	T2	T3	T4	SEM	Linear	Quadratic	Cubic
d 1-21								
WG (g)	581.33	603	602.73	673.57	24.68	0.52	0.91	0.82
FI (g)	977.26	998.58	967.73	1 034.82	20.52	0.50	0.62	0.47
FCR	1.69	1.67	1.62	1.65	0.426	0.71	0.81	0.81
d 22-42								
WG (g)	1 500.82	1 576.87	1 611.25	1 691.22	23.74	0.001	0.94	0.50
FI (g)	3 062.24	3 121.94	3 154.31	3 315.62	58.35	0.18	0.67	0.78
FCR	2.04	1.98	1.96	1.96	0.03	0.46	0.66	0.98
d 1-42								
WG (g)	2 082.14	2 179.87	2 213.98	2 328.79	36.23	0.017	0.88	0.60
FI (g)	4 042.50	4 120.52	4 122.04	4 350.44	58.45	0.10	0.51	0.55
FCR	1.94	1.89	1.86	1.87	0.03	0.38	0.67	0.97

SEM= Standard error of the mean.

sing ALIMET® supplementation levels in the diets on 21 days of age **(table IV)**. Moreover, none of samples had shown *Salmonella*. The antibodies titer of ND, AI, and IB increased (p≤0.02) linearly with increasing ALIMET® supplementation levels in the diets on 21 days of age **(table V)**. Similar trend was acquired in antibody titer of IB on 42 days of age (p= 0.006). No significant differences were observed in other parameters.

DISCUSSION

The results of dietary supplementation of Met vary depending on dietary conditions (Vazquez-Anon *et al.*, 2006b), level of supplementation (Sauer *et al.*, 2008), arginine-to-lysine ratio (Ribeiro *et al.*, 2005), or level of cysteine and choline chloride (Daenner and Bessei, 2003; Pillai *et al.*, 2005). In agreement with the results of the present study, the graded inclusion of DL-Met and ALIMET® caused a significant increase in WG in broiler chickens with no differences among the Met products (Daenner and Bessei, 2003; Abdel-Maksoud *et al.*, 2010; Saki *et al.*, 2011). The young broiler chickens have maternal reserve during the starter phase could supply broiler chicken requirements for Met irrespective form diet supplementation of Met, beneficial effects of ALIMET, act such as organic acid, on intestinal microflora (Saki *et al.*, 2011), nutrients digestibility and availability to host (Adil *et al.*, 2010) are several possibilities for increased WG with similar FI and FCR. Such findings, presumably might be as a result of offering the equilibrium content of Met activity (4.7 and 3.6 g/kg for starter and grower diets, respectively) in the experimental diets. Notably, the higher BW of chickens supplemented with DL-HMB was accompanied by higher levels of plasma insulin-like growth factor-I, a potent anabolic hormone in chickens, (Willemsen *et al.*, 2011), a finding that could help to explain the greater WG observed with the graded inclusion of ALIMET® in broiler chicken diets. There are no difference between sources and level of Met on the performance of broilers (Dibner, 2003; Dibner *et al.*, 2004; Liu *et al.*, 2007; Abdel-Maksoud *et al.*, 2010) and the supplementation of broiler diets with dry DL-Met or liquid HMB had no effect on FCR (Motl *et al.*, 2005), a finding that is in agreement with the current study.

No differences were acquired in the pH of the diets, ileum digesta, and excreta in the current study. Because of studies investigate effect of the inclusion of ALIMET® in diets on the pH are limited; the comparing of the results with other studies is difficult. However, these results implied that either greater buffering capacity of feed are effective in tolerate of ALIMET® addition in the diets or ALIMET® has limited ability to change pH of the diets unlike organic acid (Saki *et al.*,

Table III. **The pH of the diets, ileum contents, and excreta in response to the diets at different ages** (pH de las dietas, contenido del íleon y excretas en respuesta a las dietas a diferentes edades).

	Treatments					p-value		
Item	T1	T2	T3	T4	SEM	Linear	Quadratic	Cubic
d 21								
Diet	8.21	8.10	7.92	7.82	0.14	0.37	0.95	0.97
Ileum	6.53	6.50	6.23	6.04	0.09	0.42	0.65	0.68
Excreta	7.94	7.91	7.86	7.74	0.08	0.46	0.81	0.94
d 42								
Diet	8.10	8.07	8.04	7.93	0.04	0.19	0.64	0.83
Ileum	6.74	6.57	6.46	6.25	0.08	0.32	0.88	0.78
Excreta	8.08	7.93	7.89	7.86	0.05	0.18	0.61	0.84

SEM= Standard error of the mean.

Table IV. **The effect of the diets on ileum microflora (Log10 CFU/g of digesta) on days 21 and 42** (Efecto de las dietas sobre la microflora del íleon (Log10 CFU/g de digesta) en los días 21 y 42).

	Treatments					p-value		
Parameters	T1	T2	T3	T4	SEM	Linear	Quadratic	Cubic
d 21								
TNAB	9.12	8.80	8.10	7.59	0.20	0.001	0.62	0.50
Coliforms	5.74	5.62	5.52	5.29	0.13	0.27	0.84	0.90
LAB	1.36	1.51	1.44	1.65	0.06	0.17	0.77	0.38
Salmonella	Negative	Negative	Negative	Negative	NA	NA	NA	NA
d 42								
TNB	9.27	9.05	8.96	8.92	0.09	0.21	0.65	0.94
Coliforms	5.51	5.40	5.32	4.99	0.09	0.35	0.49	0.69
LAB	1.37	1.42	1.56	1.56	0.07	0.37	0.88	0.75
Salmonella	Negative	Negative	Negative	Negative	NA	NA	NA	NA

SEM= Standard error of the mean. TNBA= Total number of aerobic bacteria. LAB= Lactic acid bacteria. NA= Not applicable.

Table V. The effect of the diets on serum antibody (mg/dl) of broiler chickens at different ages (Efecto de las dietas sobre los anticuerpos séricos (mg/dl) de pollos de engorde a diferentes edades).

	Treatments					p-value		
Parameters	T1	T2	T3	T4	SEM	Linear	Quadratic	Cubic
d 21								
ND	667.29	707.93	735.16	762.17	12.92	0.004	0.70	0.87
AI	404	412.03	425.48	470.98	10.52	0.020	0.29	0.73
IB	154.95	163.31	181.13	201.52	6.65	0.005	0.53	0.87
d 42								
ND	1 425.60	1 440	1 455.52	1 439.56	7.62	0.45	0.38	0.66
AI	985.34	1 039.57	1 007.87	1 022.50	19.03	0.68	0.65	0.50
IB	400.39	430.65	448.11	499.07	13.42	0.006	0.59	0.59

Standard error of the means (SEM). Newcastle Disease (ND). Avian Influenza (AI). Infectious Bronchitis (IB).

2011) and afterward. It is demonstrated that the inclusion of organic acid in the diets had higher potential to reducing harmful bacteria in feed (Florou-Paneri *et al*., 2001) and intestinal (Saki *et al*., 2011) in favor of beneficial bacteria. Alternatively, adding organic acids in diets decrease bacteria in the small intestine and cecum (Adams, 2004) due to pH reduction (Thompson and Hinton, 1997). This can be true for ALIMET® addition in the diets, but the mode(s) of action ALIMET is unclear. These findings are agreed with the results of current study, where the inclusion of ALIMET® in the diets led to linear decrease in total number of aerobic bacteria on day 21. However, it must be noted that diminishes in bacteria counts weren't accompanied with the pH reduction in the present study. Dietary supplementation with Met increased antibody titer and has beneficial effects on the immunity system (Swain and Johri, 2000; Al-Saffar and Rose, 2002; Cunningham-Rundles *et al*., 2005; Mirzaaghatabar *et al*., 2011). The results were also established in the present study by rising serum antibodies. These results indicate that serum antibodies are sensitive to changes in Met supplementation, as reported by Mirzaaghatabar *et al*. (2011). The Met has a role in amino acid profile of antibodies molecules. Nutrient deficiencies are particularly deleterious to the immunity system when they occur early in life (Zulkifli *et al*., 1994), thus increasing Met supply could help to the fortification of immune system.

CONCLUSIONS

The results of present study point out increasing levels of ALIMET inclusion in broiler chicken diets results in improvement in WG, serum antibodies titer, and intestinal beneficial bacteria in favor of harmful ones. Improvement in Infectious Bronchitis antibody titer is highlight and the effect of ALIMET in reduction of ileum harmful bacteria is over drop of pH.

ACKNOWLEDGEMENT

The authors would like to thanks for financial supports of Agricultural Research Center of Qom-Iran. This work was supported by the Agricultural Research Center of Qom Fund, 2013, Iran under Grant Nº 14-04-13-93030.

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