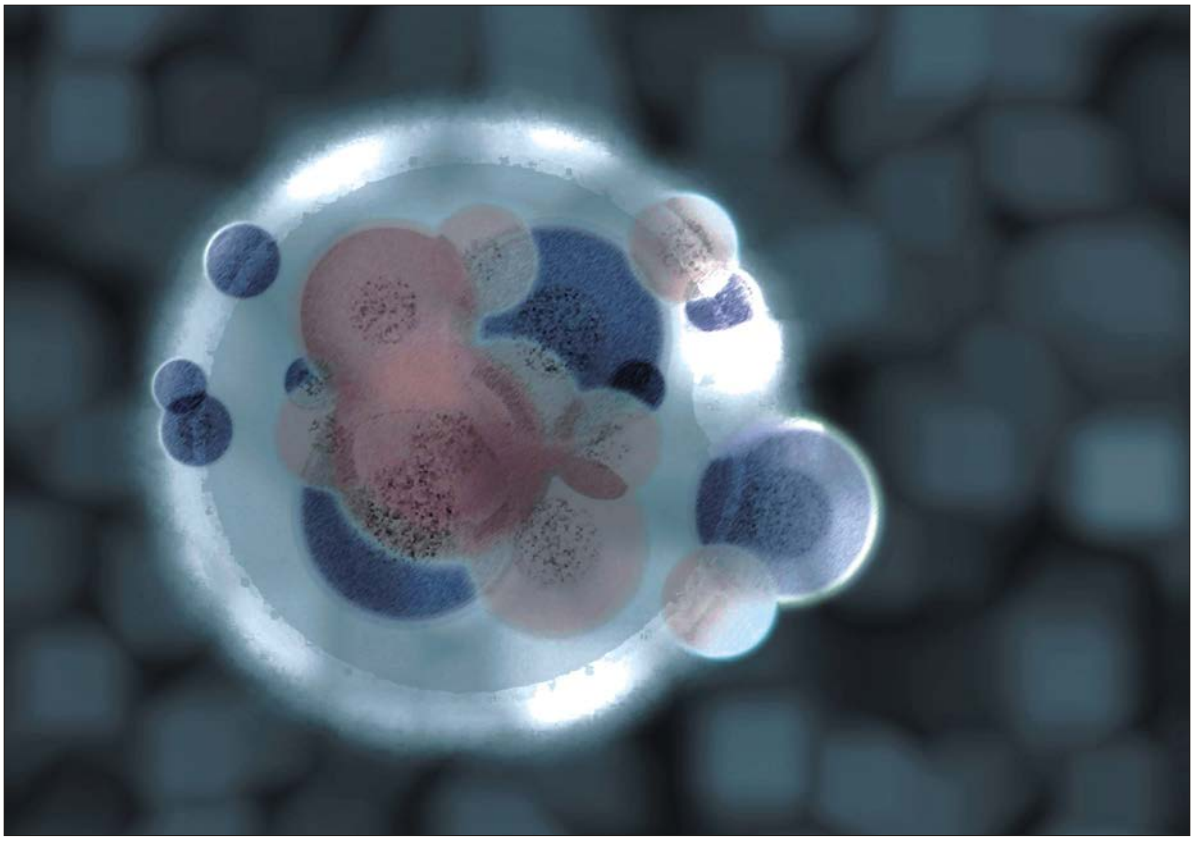

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Effects of Zinc-methionine and Feed Restriction on Performance, Immunocompetence and Gut Content Osmolarity of Broilers Challenged with a Mixed Coccidial Infection

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Abstract: An experiment was conducted in which 378 broilers received a basal diet (74 mg kg^{-1} zinc) supplemented with 0, 40 or 60 mg Zn kg^{-1} as zinc-methionine (Zn-Met) and with or without early Feed Restriction (FR) to investigate their effects on performance, humoral immunity, intestinal immune responses and gut content osmolarity especially in coccidiosis condition. Chicks consumed feed *ad libitum* or were restricted on alternate days from 11 to 18 day of age. To simulate a coccidiosis challenge, on day 28 of age the chickens were inoculated with a mixed culture of *E. tenella* and *E. acervulina* via drinking water. Antibody response to Sheep Red Blood Cell (SRBC) was determined on day 21. The immunoglobulin A (IgA) content was quantified in sera and mucous membrane tissue of intestine. Ileum and cecum content were measured for osmolarity at 21 and 42 day of age. Zn-Met could not affect average daily gain and feed intake but FR birds that dietary supplemented with 40 or 60 mg kg^{-1} Zn-Met had significantly ($p<0.01$) lower feed conversion ratio on 7 to 42 day period comparing to level 0 mg kg^{-1} of Zn-Met. Antibody response to SRBC increased by 60 mg kg^{-1} Zn-met supplementation and administration of FR ($p<0.01$). Sera IgA content increased after the coccidio-infection which its amount either in sera or gut tissue increased by adding Zn-Met to diet. Also, FR led to more IgA production ($p<0.01$). Variations in osmolarity of ileum and cecum content were similar and they were significantly lower in FR group on day 42, after the coccidiosis challenge. In conclusion, FR early in life enhanced immunity and gut condition later in life. Dietary supplementation with Zn-Met, in a level higher than common practical level, enhanced immunocompetence but not performance.

Key words: Broilers, zinc-methionine, feed restriction, immunity, immunoglobulin A, osmolarity

INTRODUCTION

Understanding the nuances of nutrition and immunity is important for optimising bird health and productivity because appropriate nutrition may aid in minimising the incidence of diseases by enhancing immunity (Klasing, 2007). According to recent study in the area of nutritional modulation of immune function, it is becoming apparent that nutrient needs for immunity do not coincide with those for growth or skeletal tissue accretion. The nutrients along with this property have been reviewed by Kidd (2004) including dietary energy and protein contents, betaine, cholin, vitamin E and A, Zinc, Manganese, etc. The Feed Restriction (FR), as limitation of energy and protein content of diet, administrated to chicks could affect the immunocompetence. In experimental infections with *Eimera tenella* (Zulkifli *et al*., 1993) and *E. coli*. Boa-Amponsem *et al*. (1997) the FR had a beneficial impact on resistance against these pathogens. Besides, FR could elevate antibody response to Sheep Red Blood Cell (SRBC) (Khajavi *et al*., 2003). However, its effect on immunoglobulin A (IgA), as an immunoglobulin against mucousal pathogens, has not been completely studied. Also, this is of interest to examine the FR for its possible effect on reduction of osmolarity and moisture of gut content in coccidiosis condition, because the reduction of moisture of litters may cause to enhance broiler status and decrease lesions which are induced by wet litters.

It has been reported that zinc levels of chick diet in practical situations are not low enough to induce immune changes (Pimentel *et al*., 1991a). In addition, it has been suggested that the immune response is less sensitive to Zn intake than growth of chicks (Pimentel *et al*., 1991b). However, some studies have reported that supplementation of diets, which contained normal level of Zn, with zinc-methionine (Zn-Met) had a beneficial effect on some different immune responses including cellular and humoral immunity (Hudson *et al*., 2004; Kidd *et al*., 2000; Virden *et al*., 2002). Although, some

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other researchers have reported in disagreement. Some of these studies have been reviewed by Kidd *et al.* (1996) who suggested; in order to determine zinc or Zn-Met requirements for broiler chick immune responses different criteria may be necessary. It's possible that much more supplemental Zn could improve some other criteria of immunity. Thus another objective of this study was to evaluate the impact of supplemental Zn-Met in diets containing practical level of Zn according to NRC (1994) recommendations on immune responses.

MATERIALS AND METHODS

Diet, birds and experimental design: This study was carried out in experimental farm of Isfahan University of Technology, Isfahan, Iran during September 2005 and April 2006. A total of 378 male and female seven day-old broiler chicks (Ross 308) were randomly assigned to 18 floor cages. Chicks were fed a basal diet supplemented with 0, 40 or 60 mg Zn kg^{-1} as Zn-Met which was administrated *ad libitum* (AL) or with feed restriction. The Zn content of basal diets (entire of ingredients except additive Zn-Met) was measured by atomic absorption spectrophotometer. The starter and grower diets contained 74 and 66 mg Zn kg^{-1}, respectively. The used Zn-Met (from Weifang Sunwin Chemical Company) contained 18.39% Zn and 43.51% methionine (analyzed by company). Dietary methionine content differences between treatments (come from supplemental Zn-Met) were adjusted by additional D-L methionine. A complete random design was used in 3×2 factorial arrangement with 3 replicates during the 7 to 42 day period. The diets were formulated to meet NRC (1994) nutrient requirements (Table 1). Except for the duration of the FR, the chickens were fed AL. The FR program involved removing feeders on day 11, 13, 15 and 17 beginning at 08:00 a.m. to 08:00 a.m. of the following day. The starter diet was given until day 21 and grower diet offered from 21 to day 42. Diets contained no coccidiostats.

Table 1: Ingredients and calculated nutrient content of diets

	Day	
Ingredients (%)	7 to 21	21 to 42
Corn	55.56	64.23
Soybean meal (44%)	34.04	29.39
Fish meal	3.50	1.00
Fatty acid	3.50	2.00
Dicalcium phosphate	1.01	1.12
Oystershell	1.39	1.39
D-L Methionine	0.14	0.03
Vitamin premix[A]	0.25	0.25
Mineral premix[B]	0.25	0.25
NaCl	0.24	0.22
Variable[C]	0.12	0.12
Energy (KCal ME kg^{-1}) and calculated nutrient content (%)		
Energy (KCal ME kg^{-1})	2991.00	2990.00
Crude protein	21.52	18.67
Lysine	1.25	0.93
Methionine	0.54	0.37
TSAA[D]	0.87	0.67
Calcium	0.93	0.81
Available phosphorus	0.42	0.37
Zn (mg kg^{-1})[E]	74.00	66.00

[A]Vitamin premix provides the following per kilogram of diet: vitamin A 8800 IU; cholecalciferol 3300 IU; vitamin E 16.53 IU; vitamin B 0.023 mg; riboflavin 8 mg; niacin 33 mg; pantothenic acid 35 mg; menadione 1.5 mg; folic acid 0.8 mg; thiamin 3 mg; pyridoxine 2.7 mg; biotin 0.25 mg; ethoxyquin 125 mg. [B]Mineral premix provides the following in milligrams per kilogram of diet: Mn, 55; Zn, 50; Fe, 50; Cu, 5; Se, 0.1; I, 1.5. [C]Variable amounts of zinc-methionine plus washed builders sand among the treatments. [D]TSAA: Total sulfur amino acids. [E]Zn content of basal diets (entire of ingredients except additive Zn-Met) was measured by atomic absorption spectrophotometer

Humoral immune assay: At day 16 of age two chickens per pen were selected randomly for intraperitonal injection with a 1.0 mL suspension of SRBC (diluted with PBS, pH 7.4 by 5% vol/vol). Five days later, those two birds were bled and antibody response was measured by microagglutination method (Wegmann and Smithies, 1966). Antibody titre was expressed as $\log_2$ of the reciprocal of the last dilution in which there was agglutination (Ambrose and Donner, 1973).

ELISA experiments: A gout anti-chicken IgA ELISA kit (Bethyl co. E30-130) was used to quantify the IgA content of sera and duodenal mucous membrane tissue. On day 21 and 42 of age two birds per pen were slaughtered to get blood samples, then the blood serum was used for ELISA test. Also, on day 42 a 3 to 4 cm in length section of duodenum (near the duodenal loop) was taken from those couple of birds. Samples were stored at -20°C until analyze time. The sections of duodenum were unfroze at 3 to 4°C then mucous membrane tissue was removed. About 0.4 g tissue was suspended in 1.6 mL cold PBS (pH 7.4) and its extract was used for ELISA test of IgA. There were differences in water content of those 0.4 g fresh tissues among the samples therefore, to adjust data for this difference, IgA amounts were expressed per gr protein of mucous membrane fresh tissue. The protein measurement was based on the standard folin method using the spectrophotometer (Lowry *et al.*, 1951).

Parasites and experimental infection: At day 28 of age, all birds were inoculated orally with a mixed culture of *Eimeria tenella* and *Eimeria acervulina* to simulate a coccidiosis challenge by 8.5×10^3 and 7.5×10^3 oocysts per chicken, respectively.

Osmolarity and moisture of gut content: Ileum and cecum contents were collected from two chickens per pen on day 21 and 42. Ileum was removed from 6 cm down stream of Meckel's diverticulum to 6 cm up stream of ileosecal

valve and its content was collected. Both cecums were sampled. Some of each sample was dried at 75°C to moisture evaluation. The rest of each sample was diluted with deionesed water then their extracts were used to osmolarity quantification by digital osmometer (vapor pressure osmometer, 5520 Vescore, USA). Data was adjusted for added water.

Statistical analysis: Data was analyzed using the GLM procedures of SAS Institute (1999). Comparisons among the means and interactions were made using Duncan's multiple range tests (Duncan, 1955) and Ls means procedure, respectively. The means differences were considered significant at a probability $p<0.05$.

RESULTS

Performance: The addition of supplemental Zn-Met in diet, as the main effect, resulted in no significant effect on the performance parameters including Average Daily Gain (ADG), Average Daily Feed Intake (ADFI) and Feed Conversion Ratio (FCR) (Table 2). However, in 7 to 21 day period not restricted chicks which received 40 mg kg^{-1} Zn-Met had different FCR comparing to those which did not receive Zn-Met. Also, FR birds that were dietary supplemented with 40 or 60 mg kg^{-1} Zn-Met had significantly ($p<0.01$) lower FCR in 7 to 42 day period comparing to those received 0 mg kg^{-1} Zn-Met.

As shown in Table 2, FR chickens had a lower ADG in 7 to 21 day period but in 21 to 42 day period they had no significant difference with AL group. Also, in this period FR chickens could consume food as much as AL group. However, at final day (day 42) the Body Weight (BW) of FR chickens was significantly lower than AL ($p<0.05$). And FCR of FR chickens was enhanced in 7 to 21, 21 to 42 and 7 to 42 day periods ($p<0.05$, $p<0.04$ and $p<0.01$, respectively) (Table 2).

Antibody response to SRBC: On day 21, at the end of restriction period, FR birds showed higher ($p<0.01$) antibody production against SRBC than AL group (Table 3). Diet supplementation with 60 mg kg^{-1} Zn-Met increased antibody response, although 40 mg kg^{-1} level could not enhance antibody titre compared with unsupplemented birds (Table 3).

Intestinal and sera IgA: The comparison of IgA content of serum between day 21 and 42 showed that the level of immunoglobulin was elevated with the growth of birds and pathogen exposure which its mean at all was 171 and 769 μg kg^{-1} on day 21 and 42, respectively. As shown in Table 3, IgA content of serum on day 21 and 42 high significantly ($p<0.001$) was higher for both levels of dietary supplemental Zn-Met than unsupplemented group. Also, the amount of intestinal IgA, measured in mucous membrane tissue, on day 42 had the same status. Birds in AL group produced significantly ($p<0.01$) lower amounts of immunoglobulin A in their blood serum on both 21 and 42 days of age as well as in their mucous membrane tissue of intestine (Table 3).

Table 2: Effect of Zn-Met and feed restriction on performance parameters

Treatments		7 to 21 day			21 to 42 day			7 to 42 day			Day 21	Day 42
Zn-Met (mg kg^{-1})	FR[A]	ADG	ADFI	FCR	ADG	ADFI	FCR	ADG	ADFI	FCR	BW	BW
		(g day^{-1})									(g)	
0	+	25.0[B]	38.5	1.54	44.0	110.6	2.49	35.7	79.0	2.21	492	1360
0	-	31.9	47.8	1.51	44.9	111.4	2.51	39.0	84.7	2.17	600	1480
40	+	27.3	39.2	1.43	46.4	107.2	2.29	38.2	77.5	2.03	530	1450
40	-	29.9	49.3	1.65	44.5	108.7	2.46	37.9	83.7	2.20	574	1442
60	+	25.1	38.4	1.52	44.2	104.0	2.32	37.3	75.6	2.02	490	1370
60	-	32.6	51.3	1.57	44.4	111.2	2.53	39.4	86.4	2.19	612	1484
SEM		1.2	1.2	0.04	1.6	2.8	0.07	0.98	1.9	0.04	21	39
Main effects (Zn-Met, mg kg^{-1})												
0		28.4[a]	43.2[a]	1.52[a]	44.4[a]	111.0[a]	2.50[a]	37.4[a]	81.8[a]	2.19[a]	546[a]	1420[a]
40		28.6[a]	44.2[a]	1.54[a]	45.5[a]	107.9[a]	2.38[a]	38.1[a]	80.6[a]	2.11[a]	552[a]	1446[a]
60		28.9[a]	44.9[a]	1.55[a]	44.3[a]	107.6[a]	2.42[a]	38.3[a]	81.0[a]	2.11[a]	551[a]	1427[a]
FR												
+		25.8[b]	38.7[b]	1.50[a]	44.9[a]	107.3[a]	2.36[b]	37.1[a]	77.4[b]	2.08[b]	504[b]	1393[b]
-		31.5[a]	49.5[a]	1.57[a]	44.6[a]	110.4[a]	2.50[a]	38.8[a]	84.9[a]	2.18[a]	596[a]	1469[a]
SEM		0.8	1.4	0.02	0.6	1.1	0.34	0.4	1.2	0.02	14	18
Source (Probability>F)												
Zn-Met		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FR		***	***	*	NS	NS	*	*	***	**	***	*
Zn-Met×FR		NS	NS	*	NS	NS	NS	NS	NS	*	NS	NS

[A]Administration of feed restriction: (+) feed restricted group and (-) *ad libitum* group. [B]Values are least square means of variables. [C]Zn-Met: Zinc-methionine. FR: Feed restriction. ADG: Average daily gain. ADFI: Average daily feed intake. FCR: Feed conversion ratio. BW: Body weight. SEM: Standard error of means. [D]a-bValues within variables with no common superscripts differ significantly ($p<0.05$). NS: Not Significant. *$p< 0.05$. $p< 0.01$. ***$p< 0.001$

Table 3: Effect of Zn-Met and feed restriction on antibody titre, IgA amounts and osmolarity of ileum and secum contents

Treatments			Sera	Sera	Intestine[B]	Osmolarity of gut content on day 21		Osmolarity of gut content on day 42	
Zn-Met ($mg\ kg^{-1}$)	FR	Antibody response to SRBC[A]	IgA on 21 day	IgA on 42 day	IgA on day 42	Ileum	Cecum	Ileum	Cecum
			($\mu g\ mL^{-1}$)		($\mu g\ g^{-1}$ p)	mOsm kg^{-1}			
0	+	3.67	144	683	24.28	481	439	463	405
0	-	3.33	120	569	20.23	470	447	531	466
40	+	4.67	229	1006	35.76	453	444	458	421
40	-	3.66	166	733	26.05	400	411	481	494
60	+	5.67	197	867	30.81	462	413	462	404
60	-	4.64	172	757	26.91	470	437	505	450
SEM		0.33	10	48	1.71	22	14	22	15
Main effects (Zn-Met, $mg\ kg^{-1}$)									
0		3.50^{b}	132^{b}	626^{b}	22.26^{b}	476^{a}	443^{a}	497^{a}	436^{a}
40		4.17^{b}	198^{a}	870^{a}	30.91^{a}	427^{a}	427^{a}	470^{a}	457^{a}
60		5.17^{a}	184^{a}	812^{a}	28.86^{a}	466^{a}	425^{a}	484^{a}	427^{a}
FR									
+		4.67^{a}	190^{a}	852^{a}	30.28^{a}	465^{a}	432^{a}	461^{b}	410^{b}
-		3.89^{b}	153^{b}	686^{b}	24.40^{b}	447^{a}	432^{a}	506^{a}	470^{a}
SEM		0.22	9	37	1.33	9.9	5.9	10	9.4
Probability>F									
Zn-Met		***	***	***	***	NS	NS	NS	NS
FR		**	***	***	***	NS	NS	*	***
Zn-Met ×FR		NS	NS	NS	NS	NS	NS	NS	NS

[A]Data for antibody response to sheep red blood cell (SRBC) are as log2 of the reciprocal of the last dilution in which there was agglutination. [B]Amounts of immunoglobulin A (IgA) was measured in mucous membrane tissue of intestine and expressed per gr protein of fresh tissue

Osmolarity and moisture of gut content: The osmolarity of ileum and cecume content on day 21 was not affected by any experimental components (Table 3). On day 42, after the coccidiosis challenge, differences of the osmolarity of ileum and cecum content among the FR and AL chickens were significant which in FR chickens were lower. However, within each group (AL or FR) there was no difference between Zn-Met levels (Table 3). Variation of data for moisture of gut content was approximately similar to osmolarity but probability levels were about $p<0.07$ to $p<0.08$.

Interactions: There was no significant interaction between Zn-Met×FR in none of the measured parameters except for FCR. There was a significant interaction between Zn-Met and FR for FCR of 7 to 42 day period. The FR birds that were dietary supplemented with 40 or 60 $mg\ kg^{-1}$ Zn-Met had significantly lower FCR in 7 to 42 day period comparing to level 0 $mg\ kg^{-1}$ of Zn-Met. We were looking for a significant interaction especially in immune response and amount of immunoglobulin production which did not exist.

DISCUSSION

This study indicated that dietary supplemental Zn-Met, as the main effect, had no significant effect on the performance parameters. This is in agreement with those reported by Kidd *et al.* (1992, 1993), who added Zn-Met to dams and their progeny diet but reported no effect on BW and FCR as well as Sandoval *et al.* (1999), who observed FCR was not affected by levels or source of Zn. However, in present study FR birds in 7 to 42 day period had lower FCR when received 40 or 60 $mg\ kg^{-1}$ Zn-Met comparing to those received no Zn-Met. Kidd *et al.* (1994a) observed an improvement in body weight gain of turkey at 3 weeks of age by Zn-Met supplementation. But in another experiment with turkey, Kidd *et al.* (1994b) reported no influence of supplemental Zn-Met on BW and feed/gain ratio on day 21. Recently Jahanian *et al.* (2008) reported that weight gains of broilers were increased by raising supplemental Zn level from 40 to 80 $mg\ kg^{-1}$ in 5 and 6 weeks of age and dietary Zn-Met supplementation affected FCR of first week and body weight gain of chickens.

Malabsorption of some nutrients is reported during the coccidiosis (Girdhar *et al.*, 2006) especially for Zn, oleic acid and Ca (Scott *et al.*, 1982) as well as amino acids, carbohydrates, vitamin E and methionine (Allen and Fetterer, 2002; Fetterer *et al.*, 2003). Thus, an assumable reason for FCR improvement of FR chickens, described in results, might be duo to effect of extra obtained Zn which is necessary for so many diverse enzymatic reactions and other physiological processes (Sandoval *et al.*, 1998) and assists metabolism to be more efficient. However, AL group received more Zn and are preferable to exposes improvement in FCR. This is the interaction which is required to more research about.

Consistent with ours, Kidd *et al.* (1992, 1993) reported improvement in first antibody response to SRBC and *Sallmonella pullorum* when dams and chicks were fed 40 $mg\ kg^{-1}$ supplemental Zn with basal diet containing

100 mg Zn kg^{-1} but they found no difference in source of Zn (ZnO or Zn-Met). However, already Pimentel *et al.* (1991a) had reported that supplementation of practical or semipurified diets with Zn (from ZnO or Zn-Met) had no impact (neither Zn level nor Zn source) on immune responses when evaluated in terms of antibody response to SRBC, human gama globulin or delayed hypersensitivity to phytohemagglutinin-P in broiler chickens. But the point is that, the diets which were formulated in that research contained up to 88 mg Zn kg^{-1} whereas, researchers who reported improvement in immunity have used the higher levels of dietary supplemental Zn (higher than 100 mg kg^{-1}). Moreover, another criterion of immune response in present study was the IgA content of sera and mucous membrane tissue of intestine. These measurements indicated that diet supplementation with 40 or 60 mg Zn kg^{-1}, led to more IgA production both in sera and intestine. Thus the overall results of immune responses suggest the same opinion as Leeson's (2005): the requirement of broilers for zinc to maximize immunocompetenc is more than NRC (1994) recommendation which is set by researches that are more than 10 years old and do not represent the needs of modern strains of commercial poultry.

Another important aspect of present findings is the significant effect of FR on antibody response to SRBC which is in agreement with those reported by Khajavi *et al.* (2003) who found that FR increased antibody response of broiler chickens under heat stress. Also, Zulkifli *et al.* (1994, 2000) reported that early FR increased antibody response to Newcastel virus. However, already Zulkifli *et al.* (1993) had not observed elevation in antibody response to SRBC with food restriction by 60% feed intake of AL. Furthermore, we observed a significant effect of the FR to elevate the IgA levels in both sera and intestinal tissue. It is likely that positive effect of FR on resistance to coccidiosis (Zulkifli *et al.*, 1993) is because of its impact on elevation of immunoglobulin production.

No literature was found about evaluation of FR effects on gut content osmolarity especially in coccidiasis condition to compare with. In this research the FR early in life showed a potential to decrease osmotic stress on epithelial cells by reducing the osmolarity and moisture of gut content later in life. According to our observations on intestinal morphology of these birds, it's likely that FR effect on later gut content osmolarity is related to morphological changes of intestinal villus especially expanding villus surface area. However, more research is needed to confirm this result and fully understand FR effects on moisture and osmolarity of intestinal content. The importance of this effect is that; the moisture reduction of droplets may decreases lesions which are induced by wet litters (e.g., Hock Scabs and Breast Burn).

According to aforesaid issue, present findings suggest that the FR is a good management tool in elevating immunocompetence of broilers in coccidiosis condition. Boa-Amponsem *et al.* (1997) has similar suggestion which reported that FR had a positive effect on resistance of white leghorn chickens to *E. coli*. However, in practical area the recommendation of FR to enhance the immunocompetence in coccidiosis condition might be uncertain because in coccidiosis condition the need of common nutrients would be increased. On the other hand, some nutrients will have malabsorption. Therefore, the FR can cause a complexity in this issue. Consequently, performance, the main goal of production, may be affected.

About the results in IgA measurement it's necessary to be described; general comparison of the levels of IgA among 21 and 42 day of age indicated an elevation after the coccidial infection, nevertheless some of this increment might be due to the growth of chickens. This growth effect on immunoglobulin production has been reported by Yun *et al.* (2000b), who observed level of immunoglobulins (IgA, IgM and IgG) in the circulating and intestine secretions elevated by the growth of birds. Yun *et al.* (2000a) at the review of intestinal immune responses to coccidiosis, suggested that the level of IgA in bile may not correlate with its level in infected areas of intestine hence, in this study IgA amounts are evaluated in the intestinal mucous membrane tissue instead of in bile. Furthermore, recently attention has been paid to passive immunoprophylaxis by orally administering the IgA that it could benefits especially for young broilers because of its fast effectiveness (Wieland *et al.*, 2006). This function reveals the importance of IgA role in protection against parasite antigens.

CONCLUSION

The observed results on immune responses suggest that dietary supplementation with Zn-Met, in a level higher than common practical level, enhanced immunocompetence but not performance. Also, suggest that the requirement of broilers for zinc to maximize immunocompetenc is more than NRC recommendation. Furthermore FR early in life enhanced immunity and gut condition later in life. According to the observed interaction between Zn-Met level and FR on FCR the more precise evaluation of this interaction would give the more clear view.

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