Effects of Exogenous Enzymes and *Salix babylonica* L. Extract on Cellular Immune Response and its Correlation with Average Daily Weight Gain in Growing Lambs[#]

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ABSTRACT

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The aim of this study was to determine the effects of exogenous enzyme (EZ) and *Salix babylonica* L. (SB) extract on cellular immune response, and its correlation with average daily weight gain (ADG). Twenty Suffolk lambs, 6-8 months of age and average live weight of 24 ± 0.3 kg, were used in a trial which lasted 60 days. The lambs were distributed into 4 groups of 5 lambs each and housed in individual 1.5x1.5 m cages in a completely randomized design. The treatments were: (i) Control; lambs consuming basal diet (BD) only; (ii) EZ; lambs consuming BD plus 10g of EZ (ZADO®); (iii) SB; lambs consuming BD plus 30 mL of SB, and (iv) EZSB; lambs consuming BD plus 10g EZ and 30 mL of SB. Blood samples were collected on days 0, 15, 30, 45 and 60 and analysed for helper T lymphocytes, cytotoxic T limphocytes, granulocytes and monocytes by flow cytometry. Treatments had no effect on parameters measured, but day of sampling had linear and cubic effects on helper T lymphocytes, granulocytes and monocytes ($P<0.01$) and cubic effects on cytotoxic T lymphocytes ($P<0.01$). The results suggest that EZ and SB have immunosuppressant effects in the first 15 days, after this effect were immunosuppressive on cytotoxic T lymphocytes and granulocytes, for monocytes the effect was immunostimulant. No there were correlation between ADG and cellular immune response in this experiment.

Key words: Exogenous enzyme, *Salix babylonica* L. extract, Immune response, Performance, Lambs.

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INTRODUCTION

There is a complex tripartite interaction between malnutrition, immune system and infectious diseases (Mitchell *et al.*, 2003). Deficiency of macro- and micro-nutrients causes dysfunction of the immune system (Mitchell *et al.*, 2003; Chandra, 2004; Wintergerst *et al.*, 2007). These deficiencies increase the vulnerability of animals to disease which is exacerbated by malnutrition both altering defense mechanisms such as anatomical barriers, cellular and humoral immune responses against antigens (Calder and Kew, 2002; Maggini *et al.*, 2007). Thus, the animal is not capable to initiate an immune response and the infection might not be controlled (Erickson *et al.*, 2000; Enwonwu, 2006) leading the animal to under-express their productive potential.

In order to promote a more efficient use of macro and micro-nutrients contained in the diet, feed additives such as pre- and probiotics, enzymes and exogenous ionophores and fodder tree extracts have been examined in previous studies (Salem *et al.*, 2010; Salem *et al.*, 2011a; Chung *et al.*, 2012). Although there is evidence that such feed additives can directly or indirectly maintain the immunocompetence of the animal, namely by stimulating cells of the immune system or providing nutrients for these cells to function normally (Nayak, 2010; Owusu-Asiedu *et al.*, 2010).

The activity of ZADO® which is a commercial endogenous enzyme (EZ), has proved to increase the digestibility of DM, CP, NDF, ADF and the concentration of volatile fatty acids in rumen, and thus, resulting in improvements in animal productivity by increasing feed intake, ADG, feed efficiency and milk production (Arriola *et al.*, 2011; Gado *et al.*, 2011). Addition of EZ increased forage energy utilization which may have an indirect effect on the immune system under the assumptions of immune nutrition which are based in adequate utilization of dietary nutrients to promote maintenance of immunocompetence (Keith and Jeejeebhoy, 1997).

There are different types of possible measurements to investigate relationships between nutrition and immune response. Phenotyping and enumeration of immune cells by flow cytometry has proven to be accurate allowing the comparison of cell ratios and analysis of cell counts (Mitchell *et al.*, 2003). The objective was to determine effects of exogenous enzyme and *S. babylonica* L. extract on cellular immune response and to evaluate if it correlates with average daily weight gain in lambs.

MATERIALS AND METHODS

This study was conducted at the experimental farm unit of the Faculty of Veterinary Medicine of the Autonomous University of Mexico State. The handling of animals was performed according to international bioethical standards and NOM-062-ZOO-1999 (SAGARPA, 1999).

Animals and treatments

Twenty Suffolk lambs, 6-8 months of age and average live weight of 24 ± 0.3 kg, were used. Lambs were housed in individual 1.5×1.5 m cages in a completely

randomized design and the experiment was conducted for a period of 60 days. After 2 weeks of adaptation to the basal diet of 70% corn silage and 30% commercial concentrate (Purina®, Cuautitlan, Mexico) which was formulated to meet nutrient requirements (NRC, 1985), the lambs were weighed and randomly distributed into 4 groups of 5 lambs. The treatments were: (i) Control: basal diet of concentrate and corn silage; (ii) EZ: basal diet plus 10 g/day of exogenous enzyme preparations (ZADO®, Cairo, Egypt); (iii) SB: basal diet plus 30 mL/day of *Salix babylonica* L. extract, and (iv) EZSB: basal diet plus 10 g/day exogenous enzymes and 30 mL of *S. babylonica* L. extract. The daily dose of SB was given orally before the morning feeding; the EZ was mixed with 200 g of concentrate and offered 1 h before the rest to corn silage and concentrate was provided. The chemical composition of the basal diets is shown in Table 1.

Table 1. Chemical composition of the basal diet (commercial concentrate and corn silage) and levels of secondary metabolites (g/kg DM) in the *S. babylonica* L. extract

	Concentrate	Corn silage	*S. babylonica* L. extract
Chemical composition			
Dry matter[1]	880	360	
Organic matter	325	684	
Crude protein	157.1	106.2	
Ether extract	119.8	85.2	
Neutral detergent fiber	160.4	444.8	
Acid detergent fiber	27.7	111.2	
Lignin	7.6	18.0	
Ingredients of concentrate g/kg			
Corn grain flacked	200		
Corn grain cracked	260		
Sorghum grain	154		
Molasses sugar cane	100		
DDG	100		
Soya bean meal	96		
Weath bran	70		
NaCOO$_3$	10		
Mineral mixture[2]	10		
Secondary metabolites			
Total phenolics	-	-	16.4
Saponins	-	-	5.4
Aqueous fraction[3]	-	-	76.3

[1]DM expressed as g/kg fresh silage; [2]Mineral mixture: Ca, 190 g/d; P, 115 g/d; Mg, 63 g/d; Cl, 167 g/d; K, 380 g/d; Na, 70 g/d; S, 53 g/d; Co, 3.3 mg/d; Cu, 197 mg/d; Fe, 360 mg/d; Mn, 900 mg/d; Se, 2 mg/d; Zn, 810 mg/d; vitamin A, 940 (1000 IU/d); vitamin D, 165 (1000 IU/d); vitamin E, 374 (1000 IU/d); [3]The aqueous fraction contains lectins, polypeptides and starch (Cowan, 1999).

ZADO® is a powdered multi-enzyme commercially available feed additive produced from *Ruminococcus flavefaciens* by the Academy of Scientific Research and Technology in Egypt (Patent No.: 22155, Cairo, Egypt). Prior to the study, the mixture was assayed for several enzymatic activities and found to contain (/g of enzyme preparation) 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α- amylase and 29.2 units of protease activity (Gado *et al.*, 2011).

In order to prepare silage, whole corn plants (at ~70% moisture) were chopped into 1 to 2 cm pieces using a forage chopper. Silage was accomplished in a flat 10 t silo and after 2 months, it was offered for feeding.

Preparation of extract

The *S. babylonica* L. extract was prepared as described by Salem *et al.* (2011b). Briefly, fresh leaves of *S. babylonica* L. were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and chopped (1 to 2 cm) and immediately extracted in the proportion of 1 g leaf per 8 mL of solvent mixture, which contained 10 mL methanol (99.8/100, analytical grade, Fermont®, Monterrey, Mexico), 10 mL ethanol (99/100, analytical grade, Fermont®, Monterrey, Mexico) and 80 mL distilled water. Leaves were soaked and incubated in this solvent in the laboratory at 25 to 30°C for 48 to 72 h in closed flasks. After incubation, all flasks were incubated in a water bath at 39°C for 1 h and then immediately filtered and the filtrate collected and stored at 4°C for further use.

Sampling and measurements

Five ml of peripheral blood were withdrawn from each animal via jugular venipuncture into Heparin vacutainer tubes (BD tube, Monterrey, Mexico) kept in ice on days 0, 15, 30, 45 and 60 of the experiment. Blood was analysed to determine immune phenotypes by flow cytometry. The immune phenotypes determined were helper T lymphocytes (Ab mouse anti sheep CD4RPE/MCA2213PE), cytotoxic T lymphocytes (Ab mouse anti sheep CD8FITC/MCA2216F, D.F, Mexico), granulocytes (Ab mouse anti bovine CD11bFITC/MCA1425F,.D.F, Mexico) and monocytes (Ab mouse anti human CD14RPE/MCA1568PE, D.F, Mexico) with antibodies AbDSerotec®.

The animals were weighed on the day of sampling, every 15 days. The feed offered and rejected was weighed every day. Two samples of concentrate, silage and SB extract were collected weekly and stored at -20°C for later chemical analysis. Additional samples by type (*i.e.,* concentrate, silage, extract) were pooled and stored for further analysis.

Processing of samples

Each sample was processed to determine the four immune phenotypes, initially the cellular gradient was obtained using Ficoll (Limphoprep® BD) to recover white

blood cells as lymphocytes (T helper and cytotoxic), granulocytes (neutrophils, eosinophils and basophils) and monocytes (macrophages) (Macey, 2007).

Gradient

Blood sample and PBS 1 x were used to make a 1: 1 dilution. This mixture was placed in 3 mL of cold lymphoprep®, centrifuged at 1200 rpm at 4°C for 20 min, then the collected cells were placed in 2 mL of PBS 1x and centrifuged again at 1500 rpm at 4°C for 5 min (Macey, 2007).

Sample staining

After the cell gradient was processed, 20 μL of each sample was placed in a 96 well plate and centrifuged at 1500 rpm for 5 min. The supernatant was removed and 50 μL of the adequate antibody was incorporated (helper T lymphocytes, cytotoxic T lymphocytes, granulocytes, monocytes). After 30 min at 4°C incubation, 100 μL of PBS 1x was added, centrifuged again and the supernatant removed, while a secondary antibody was added and incubated for 30 min at 4°C. Then a final wash with PBS 1x was performed. The readings were conducted in a flow cytometer (FacsCalibur) (Macey, 2007).

Chemical analyses and assays

Samples of concentrate and silage were analysed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997) and Van Soest *et al.* (1991). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (sa) (AOAC, 1997; #973.18) were analysed using an ANKOM 200 Fibre Analyzer Unit (ANKOM Technology Inc., Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulphite. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined using 10 mL of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) to determine total phenolics by drying and to quantify the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont®, Monterrey, Mexico) was added to fractionate saponins (Ahmed *et al.*, 1990). The remaining solution was considered to be the aqueous fraction which contains the other secondary metabolites, lectins, polypeptides and starch (Cowan, 1999; Table 1).

Endoglucanase activity was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes liberation of one mmol of glucose/min from sodium carboxymethyl cellulose at 40°C and pH 4.5. The α-amylase was assayed by its ability to produce reducing groups from starch, which were measured by reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of

α-amylase catalyzes liberation of one mmol of reducing groups/min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by hydrolysis of dimethyl casein (DMC) and liberated amino acids were determined using 2,4,6-trinitrobenzene sulfonic acid (Lin *et al.*, 1969). One DMC-U catalyzes cleavage of one mmol of peptide bond/min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes liberation of one mmol reducing groups per hour from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

Statistical analyses

Data related to immunological parameters were analysed using the MIXED procedure of SAS (2002) with repeated measures (Littell *et al.*, 1998). The structure of the variance-covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were diet (*i.e.,* control, SB, EZ, EZSB), days of sampling (*i.e.,* 0, 15, 30, 45 and 60 of the experiment) and its linear, quadratic and cubic effects. The repeated term was sampling days, with lamb within diet as the subject. Results are reported in Tables and in text with their respective standard error of the mean. Tests of simple effects were used to partition interaction effects by diet in order to test effects of period separately for each diet using SAS. Significant differences between treatment means and time were assessed using Tukey's test at $P < 0.05$ level. Additionally, correlation analyses between ADG and each of the four immunological parameters (*i.e.,* helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes) for treatment and day of sample were performed using PROC CORR of SAS.

RESULTS

Effect of EZ on immunological parameters

Treatment resulted in no differences on the four immunological parameters measured (Table 2). Addition of this additive resulted in no effects on DMI and ADG (Table 2). Similarly, no correlations between ADG and each of the immunological parameters such as helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes were registered.

Effect of SB extract on immunological parameters

There were no differences among treatments due to incorporation of SB to the diets of lambs. The values of helper T lymphocytes and cytotoxic T lymphocytes increased in lambs offered SB compared to the control. Granulocytes and monocytes, however, decreased in lambs fed SB compared to the control (Table 2). The DMI and ADG were unaffected by the intake of *S. babylonica* extract (Table 2). The ADG was not correlated with helper T lymphocytes or cytotoxic T lymphocytes. The same trend

Table 2. Effects of exogenous enzyme preparation (EZ) and S. *babylonica* L. (SB) extract as well as their mixture (EZSB) on cellular immune response parameters, dry matter intake (DMI) and average daily gain (ADG) in growing lambs after 0, 15, 30, 45 and 60 days of the experiment (*n*=5 lambs).

	Treatment				SEM	*P*	Day of Experiment					SEM	*P*		
	Control	EZ	SB	EZSB			D0	D15	D30	D45	D60		Linear	Quadratic	Cubic
Helper T lymphocytes	7.54	7.97	7.75	7.65	0.323	0.81	6.59[b]	5.63[c]	9.30[a]	9.55[a]	7.55[b]	0.351	<0.01	0.09	<0.01
Cytotoxic T lymphocytes	40.23	38.47	40.47	41.01	0.934	0.28	37.8[b]	43.7[a]	33.4[c]	41.0[a]	44.3[a]	1.04	0.89	0.42	<0.01
Granulocytes	38.59	38.18	37.79	38.23	0.596	0.82	38.36[b]	43.26[a]	31.41[c]	38.45[b]	39.51[b]	0.568	<0.01	0.06	<0.01
Monocytes	9.87	9.8	9.2	9.48	0.366	0.55	5.86[c]	6.16[c]	9.53[b]	13.24[a]	13.14[a]	0.388	<0.01	<0.01	0.18
DMI g/d	496.4	491.61	528.84	494.04	1.65	0.96	-	-	-	-	-	-	-	-	-
ADG	104.91	105.72	100.54	122.31	0.4357	0.7108	-	-	-	-	-	-	-	-	-

[abc] Different superscripts following means within the same row and experimental factor indicate differences at $P<0.05$; SB, S. *babylonica* L. extract; EZ, exogenous enzyme; EZSB, S. *babylonica* L. extract + exogenous enzyme.

Table 3. Pearson's correlation coefficient (r) between average daily weight gain and cellular immune response parameters of growing lambs fed diet with addition of exogenous enzyme preparation (EZ) and S. *babylonica* L. (SB) extract as well as their mixture (EZSB) after 15, 30, 45, and 60 days of the experiment (*n*=5 lambs).

	Treatment								Day of experiment							
	Control		SB		EZ		EZSB		15		30		45		60	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Helper T lymphocytes	0.73	0.27	-0.12	0.88	0.77	0.23	0.58	0.42	-0.59	0.94	-0.87	0.13	-0.33	0.67	0.37	0.63
Cytotoxic T lymphocytes	-0.85	0.15	-0.60	0.40	-0.30	0.70	-0.97	0.03	0.45	0.55	-0.23	0.77	0.75	0.25	-0.21	0.79
Granulocytes	-0.88	0.12	-0.73	0.27	-0.38	0.62	-0.91	0.09	-0.64	0.37	0.30	0.70	0.39	0.61	0.04	0.96
Monocytes	0.12	0.86	-0.88	0.12	0.84	0.16	-0.06	0.94	-0.71	0.29	-0.24	0.76	0.58	0.42	-0.03	0.97

SB, S. *babylonica* L. extract; EZ, exogenous enzyme; EZSB, S. *babylonica* L. extract + exogenous enzyme.

was observed for granulocytes (r=-0.73, P=0.27) and monocytes (r=-0.88, P=0.12) as shown in Table 3. Helper T lymphocytes values were also not correlated to treatment EZSB (r=0.58, P=0.42), while they were negatively correlated to cytotoxic T lymphocytes (r=-0.97, P=0.03). Granulocytes tended to be negatively correlated (r=-0.91, P=0.09) to the same treatment.

Effect of the time of sampling on immunological parameters

The effect of time of sampling was different among treatments. Helper T lymphocytes values increased (P=0.01, linear and cubic effect), on day 30 and 45 compared with the day 15 when this value decreased. In the case of cytotoxic T lymphocytes, the values increased (P=0.01, cubic effect) on day 15, 45 and 60 and decreased on day 0 and 30. The values of granulocytes increased (P=0.01, linear and cubic effect), on day 15 compared to days 0, 30, 45 and 60 of the experiment. In accordance, monocytes values also increased (P=0.01, linear and quadratic effect) on day 45 and 60 and decreased on day 0 and 15 and 30 (Table 2). There were no correlations between some immunity parameters and ADG.

DISCUSSION

Effect of EZ on immunological parameters

There is no available literature on the impact of EZ on the immunological status in ruminants and in this study addition of EZ did not produce negative effects on immunological parameters. In a previous study, researchers did not observe adverse effects on animal health when they use 10 g of EZ per lamb/day (Salem *et al.*, 2011a).

However, enzymes are not routinely used in ruminant diets because it is generally assumed the enzyme proteins would be rapidly degraded by ruminal microbes (Beauchemin *et al.*, 1995; Beauchemin *et al.*, 2003). Addition of EZ to lamb diets did not resulted in correlations between ADG and the helper T lymphocytes and monocytes which suggests that when the animals are able to meet their nutrient requirements, they are capable of initiating immune response against antigens (Erickson *et al.*, 2000; Mitchell *et al.*, 2003; Enwonwu, 2006).

Effect of SB extract on immunological parameters

Extract of *S. babylonica* L. did not result in a detrimetal effect on immunological parameters in the present study, which was probably due to the low concentrations of secondary compounds (principally tannins and saponins) which were dosed daily to the lambs (Khalil and El-Adawy, 1994). Others studies have indicated negative effects of tannins and saponins on red and white blood cells counts resulting in anemia and immune deficiency (Adedapo *et al.*, 2005; Adedapo *et al.*, 2007; Mahgoub *et al.*, 2008). The absence of a negative effect on white blood cells in our study indicates that the SB extract is innocuous to this kind of cells. However, the same dose of SB extract used in other experiments showed anti-helmintic properties reducing intestinal worm

loads up to 40%, which represent an important effect for animal health, moreover, feed intake was not affected by extract addition as occurred in the present study (Salem *et al.*, 2011b).

We did not observe negative effects of ethanol present in the extract which in others studies has shown immunosuppressive effects by inhibition of enzymatic pathways (Hote *et al.*, 2008), therefore demonstrating that the ethanol content in the extract of *S. babylonica* L. is safe to lambs. Our results are not in accordance to those reported by Mahgoub *et al.* (2008) and Adedapo *et al.* (2007) who observed negative effects on white blood cells in sheep consuming diets rich in secondary compounds or extracts from different plants.

There were no correlations between ADG and the cytotoxic T lymphocytes, granulocytes and monocytes in SB lambs. These results suggested that when the animal presents increased immunological parameters, it cannot express its potential in production because the engulfing, presence or processing of the antigen may cause fever, emesis, diarrhea and anorexia (Grimble, 1998; Maggini *et al.*, 2007). This scenario promotes decrease in feed intake, ADG, milk production and other productive parameters (Maggini *et al.*, 2007).

There were negative (r=-0.97, P=0.03) correlations between ADG and cytotoxic T lymphocytes in EZSB group. This result suggests that when the animal present increased levels of such types of cells, it is processing an antigen which promotes a detrimental effect on ADG for the mechanism mentioned above. On the contrary, when the levels the cytotoxic T lymphocytes are low, the animal seems healthy which contribute to reach its maximal performance (Grimble, 1998; Maggini *et al.*, 2007).

Effect of the time of sampling on immunological parameters

The level of helper T lymphocytes in peripheral blood was linearly increased as the time of sampling advanced. These results indicate that treatments had negative effect (immunosuppressant) on helper T lymphocytes for the first fifteen days and then it had an immunostimulant effect on these cells, which remains for 30 days and then this effect began to diminish. Similar immunostimulant effects have been observed in fish treated with plant extracts and their products (Harikrishnan *et al.*, 2011). In ruminants, a period for adaptation while conducting nutrition experiments is very important, due to the fact that rumen micro-organisms require a minimum of 7 days to adapt to a diet. Thereafter, the micro-organisms begin to metabolize the dietary treatments (Van Soest, 1991). In this experiment, this time was evaluated in order to know the time it took to observe immunostimulant or immunosuppressant effects and also how long these effects remained. Mahgoub *et al.* (2008) evaluated the effect of secondary compounds at the beginning and at the end of the experiment, and they observed a decrement in the counts of this type of cells, nonetheless, this effect was weak at the end of the experiment.

The cytotoxic T lymphocytes augmented with time, and this increase was more

evident in the first 15 days. There was a more prolonged immunostimulant effect which suggests that the treatments had a positive effect on cytotoxic T lymphocytes. The effect on white blood cells is indirect although excessive ingestion of a wide variety of plants or their products (extracts) has been found to cause hypoproliferative or non-regenerative anemia. This cell disorder is characterized by reduced bone marrow production of all blood components in the absence of a primary disease process infiltrating the bone marrow or suppressing haematopoiesis. This process had a negative effect on neutrophils, lymphocytes, eosinophils, and monocytes (Adedapo *et al.*, 2007).

The time effect on granulocytes (neutrophils, eosinophils and basophils) was similar to cytotoxic T lymphocytes, with a stimulating effect on this cell type. These results may be due to the effect of extract saponins on cells and immune response (Erickson *et al.*, 2000; Harikrishnan *et al.*, 2011). Reports indicate that the function of neutrophils, eosinophils and basophils depends upon adequate consumption of macro and micronutrients; thus, malnutrition contributes to immune suppression (Mitchell *et al.*, 2003).

Regarding monocytes, our results suggest an immunostimulator effect because the animals them to 14 days have the capacity to metabolize and use any compounds presents in the diet. These results confirm that time is an important issue while feed additives are used and the period of adaptation to a particular diet as well (Van Soest, 1994).

CONCLUSIONS

The results suggest that exogenous enzyme and *S. babylonica* L. extract have immunostimulant effect the first 15 days, after these feed additives have immunosuppressive effects principally on cytotoxic T lymphocytes and granulocytes. The results indicate that EZ and SB as used in this experiment did not affect cellular immune response, thus, they might be used in lamb nutrition practices promoting growth performance without altering animal health. Both feed additives produced similar results supporting the use of *Salix babylonica* L extract as a suitable alternative when people cannot access to EZ.

The effect of the treatments throughout time ought to be considered in animal nutrition experiments. Studies evaluating the immune response might favour estimations of the effect of feed additives after the initial fifteen days.

ACKNOWLEDGMENTS

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