

ANIMAL RESEARCH PAPER

Influence of exogenous enzymes in presence of *Salix babylonica* extract on digestibility, microbial protein synthesis and performance of lambs fed maize silage

K. I. VALDES¹, A. Z. M. SALEM^{1*}, S. LOPEZ², M. U. ALONSO¹, N. RIVERO³,
M. M. Y. ELGHANDOUR¹, I. A. DOMÍNGUEZ¹, M. G. RONQUILLO¹ AND A. E. KHOLIF⁴

¹ Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Toluca

² Instituto de Ganadería de Montaña (IGM) CSIC-Universidad de León, Departamento de Producción Animal, Universidad de León, E-24071 León, Spain

³ División Académica de Ciencias Agropecuarias, Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco 86280, México

⁴ Dairy Science Department, National Research Centre, El Buhoth Street, Dokki, Cairo, Egypt

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SUMMARY

Sixteen Suffolk lambs with 29 ± 2.0 kg body weight were housed in individual cages for 60 days and allotted to four treatments in a completely randomized design to determine the effect of administration of *Salix babylonica* (SB) extract and/or exogenous enzymes (ZADO[®]) on lamb performance. Lambs were fed with 300 g/kg concentrate (160 g crude protein (CP)/kg, 13.4 MJ metabolizable energy (ME)/kg dry matter (DM)) and 700 g/kg maize silage (80 g/kg CP, 11.7 MJ ME/kg DM) as a basal diet (control). Another three treatments were tested; the SB extract was administered at 30 ml/day (SB) and exogenous enzymes ZADO[®] (i.e. an exogenous enzyme cocktail in a powder form) directly fed at 10 g/day (EZ), while the last treatment contained ZADO[®] at 10 g/day + SB extract at 30 ml/day (EZSB). Lambs of the treatment EZSB had the greatest average daily weight gain (ADG) and feed conversion throughout the period of the experiment. However, during the first 30 days SB was more effective for ADG than EZ and *vice versa* during the last 30 days of the experiment. Water consumption was greater for SB, followed by EZ and EZSB compared to the control. Intakes of DM and organic matter (OM) were the highest in EZSB followed by EZ, which had the greatest neutral detergent fibre, acid detergent fibre (ADF) and nitrogen (N) intakes. The EZSB treatment had the greatest DM and OM digestibilities compared to the other treatments; however, SB had the greatest ADF digestibility. Combination of EZ and SB had the best N balance. Allantoin, total purine derivatives (PD), allantoin : -creatinine ratio, and PD : creatinine ratio were increased in EZSB compared to the other treatments. However, EZ supplementation increased uric acid concentration, whereas the microbial N (g N/day) and metabolizable protein (g N/day) were increased in EZSB *versus* the other treatments. It can be concluded that addition of 10 g ZADO[®] in combination with *S. babylonica* extract at 30 ml/day in the diet of lambs increased feed intake, nutrient digestibility and daily gain, with a positive impact on the use of N and microbial protein synthesis.

INTRODUCTION

Recently, nutritionists have been searching for natural strategies with low cost and easy application in order to improve animal performance (Durmic & Blache 2012). Antibiotics and ionophores have very

successful results for reducing energy and protein losses in the rumen (McGuffey *et al.* 2001). However, the use of antibiotics in animal feeds is facing reduced social acceptance due to the potential appearance of residues in animal products (Russell & Houlihan 2003). In addition, their use has been banned in the European Union since 2006 (Official Journal of the European Union 2003). For these

* To whom all correspondence should be addressed. Email: asalem70@yahoo.com

reasons, there is interest in using medical plants and plant extracts as alternatives, based on their potent properties and complex bioactivity (Durmic & Blache 2012). In Mexico, there are native trees that can be used as an alternative feed when forages are scarce and of poor quality in the dry season (Palma *et al.* 1995). However, the use of plants or their extracts as feed additives is restricted by their secondary compound content (Salem *et al.* 2014c): although the ideal concentrations can modify and support the utilization of nutrients in the rumen (Salem *et al.* 2014a, b, c), an inverse relationship between secondary compound concentration and animal performance has been observed (Vasta & Luciano 2011; Salem *et al.* 2011a, 2013).

Some plant extracts contain secondary metabolites with active substances and molecules, released in the rumen during fermentation, that have the ability to change the activity of rumen microbial fermentation and stimulate appetite and digestion, due to their antimicrobial activity against some ruminal bacterial species. Some of these active compounds have antioxidant, anti-inflammatory, antiseptic or antiprotozoal properties, inhibiting ruminal ammonia nitrogen (N) release (Kamel 2001; Wallace *et al.* 2002; Mejía-Hernández *et al.* 2014; Salem *et al.* 2014c). This activity is due to the action of secondary metabolites such as tannins, saponins and essential oils. Moreover, it is known that microbial protein is an important part of the N flow of post-digestive tracts in ruminants, so that the excretion of purine derivatives (PD) and flow of purine bases into the duodenum have been used as a parameter to estimate the microbial protein flow in ruminants (Balcells *et al.* 1992).

The use of exogenous enzymes in animal feed as an additive improves the nutritional value of tree foliage due to the occurrence of solubilization in the dietary fibre. Morgavi *et al.* (2000) showed that the use of exogenous enzymes has the potential to improve the quality of fodder trees used as natural additives for ruminant feeding. Exogenous enzymes can stimulate increases in the total number of viable bacteria, increasing fibre digestion and improving the ability of rumen bacteria to ingest and degrade feed and secondary metabolites. It could also increase the amount of crude protein (CP) available for microbial metabolism, which may increase fibre digestibility and the metabolizable energy (ME) density of the diet (Salem *et al.* 2012). The exogenous fibrolytic enzymes can work synergistically with exogenous rumen microbial

enzymes and thus could increase the digestion and nutritive value of fibrous diets (Morgavi *et al.* 2001).

A hypothesis was developed to explain the synergistic effects resulting from the combination of *Salix babylonica* (SB) extract and direct-fed enzyme on animal performance: optimal doses of secondary metabolic compounds from plant extracts with optimum doses of enzymes may have cumulative effects on animal performance. Rivero *et al.* (2012, 2013) tested the effect of administering 30 ml SB extract in combination with 10 g ZADO[®] (i.e. an exogenous enzyme cocktail) on the performance of Suffolk lambs. Their results indicated that ZADO[®] enzyme (EZ) and SB as single or combined feed additives promoted growth performance without altering animal health or affecting cellular immune response or blood chemistry.

Therefore, the aim of the present study was to determine the effects of administering SB extract or/and exogenous enzymes on performance of lambs fed maize silage and concentrate as a basal diet.

MATERIALS AND METHODS

Animals and treatments

Sixteen male Suffolk lambs (29 ± 2.0 kg body weight) were divided into four treatments to be fed the control diet (300 g/kg concentrate (160 g CP/kg, 13.4 MJ ME/kg dry matter (DM) + 700 g/kg maize silage (80 g CP/kg, 11.7 MJ ME/kg DM)), SB (30 ml extract/animal/day), EZ (10 g ZADO[®]/animal/day) or a mixture EZSB (10 g ZADO[®] + 30 ml extract/animal/day). The extract was administered orally to individual lambs, once daily, with a 30 ml syringe before feeding, while the enzymes were provided in 100 g concentrate DM before feeding to assure their intake. Animals were housed in individual cages and offered concentrate and maize silage (Table 1) twice daily (08:00 and 16:00 h) for 60 days, with 10 days of adaptation to the diet. Feed and water intake were recorded every day. Each animal's weight was recorded every 15 days, after feed and water removal and before adding a new feed or water (shrunk weight) to calculate the average daily gain (ADG, g/day) and feed conversion (FC, g/day). Animals were housed in individual metabolic cages for 7 days in which faeces, urine and feed samples were collected daily. Sulphuric acid (50 ml/l) was added to the urine to keep the pH <3.0 in order to prevent proliferation of bacterial pathogens that

Table 1. *Chemical composition of maize silage and concentrate mixture as well as the ingredients of the concentrate (g/kg DM)*

	Concentrate	Maize silage
Dry matter*	880	360
Organic matter	325	342
Crude protein	157	72
Ether extract	120	85
Neutral detergent fibre	160	445
Acid detergent fibre	28	111
Lignin	8	18
Ingredient of concentrate		
Maize grain flaked	200	
Maize grain cracked	260	
Sorghum grain	154	
Molasses sugarcane	100	
Distilled dry grain	100	
Soya bean meal	96	
Wheat bran	70	
NaCOOH ₃	10	
Mineral mixture [†]	10	

* Dry matter expressed as g/kg fresh feed.

[†] Mineral/vitamin premix (25) (vitamin A (12 000 000 IU), vitamin D3 (2 500 000 IU), vitamin E (15 000 IU), vitamin K (2.0 g), vitamin B1 (2.25 g), vitamin B2 (7.5 g), vitamin B6 (3.5 g), vitamin B12 (20 mg), pantothenic acid (12.5 g), folic acid (1.5 g), biotin (125 mg), niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g).

could potentially destroy any PD present in each sample collected, then samples were frozen (−20 °C) for later analysis.

The commercial enzyme product, ZADO[®] is an enzyme cocktail obtained from *Ruminococcus flavefaciens*, recently developed by the laboratory of Rumen Ecology Centre, Animal Production Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. ZADO[®] is a powdered multi-mix of cellulases, xylanases, protease and α -amylase enzymes, in addition to the related anaerobic bacteria which produce these enzymes (Khattab *et al.* 2011), coated with starch and glycol. ZADO[®] is given to animals directly before feeding and is active immediately after feeding. The main actions are on rumen kinetics and improvements in how effectively the rumen microflora can utilize feed ingredients, and should be reflected in the animal's performance in terms of either milk or meat production (Khattab *et al.* 2011; Salem *et al.* 2013).

Enzyme activities in the enzyme preparation were determined for endoglucanase (Robyt & Whelan 1972), α -amylase activity (Bernfeld 1955), protease activity (Lin *et al.* 1969) and xylanase activity (Robyt & Whelan 1972) by catalysing hydrolysis of xylan from oat spelt: the reducing groups liberated were determined using alkaline copper reagent (Table 2).

Preparation of maize silage

Whole maize plants were cut at the beginning of grain development, growth stage (GS) 71 according to the BBCH scale (Lancashire *et al.* 1991): kernels were at the blister stage and c. 16% DM. Maize plants were chopped into lengths of 0.5–1.0 cm. Plants were ensiled and compacted with a tractor, covered with black plastic and tyres, and allowed to ferment for a further 1 year before feeding to sheep in the present study.

Preparation of extract

Preparation of the extract was as described by Salem (2012). Briefly, SB leaves collected from five different willow trees were blended in a Wiley mill. One kg of leaves in 8 litres of methanol/ethanol/distilled water (10/10/80, v/v/v) was kept at room temperature for 48 h before being placed in a water bath for 60 min at 30 °C. The solution was filtered with gauze, discarding the solid fraction and liquid fraction was retained at 4 °C.

For determination of active chemical constituents, subsamples of SB (100 g) were soaked in 150 ml of methanol, acetone and hexane (1 : 1 : 1 v/v/v) solvent at room temperature for 24 h. The crude extract was filtered through Whatman No.1 and over active charcoal to remove chlorophyll. The extract was concentrated in a vacuum to 20 ml and lyophilized to obtain the dried extract: 10 ml of the extract was used in gas chromatography–mass spectrophotometry (GC–MS; Varian Saturn 2100 T 3900 GC/MS mass selective detector connected to a RTX 6890 Gas Chromatograph, NY, USA) analysis. Separation was carried out in a capillary column, RTX 5MS (50 ml/l phenyl methyl polysiloxane) 30 m long, 0.25 mm internal diameter and 0.25 μ m film thickness. The column temperature was kept at 50 °C for 6 min and programmed to increase to 300 °C at a rate of 5 °C per min. The flow rate of helium (the carrier gas) was 1 ml/min with a split vent flow of 20 ml/min.

Table 2. Composition of exogenous enzymes of ZADO®

Samples	Xylanase*	α -Amylase†	Endoglucanase‡	Protease§
ZADO®	2.3 U/g	61.5 U/g	7.1 U/g	29.2 U/g

* One unit (U) is defined as the amount (g) of enzyme needed to release 1 μ mol xylose per minute from 5 mg/ml xylan solution (pH 5.5 and 37 °C).

† One unit (U) is defined as enzyme needed to produce 1 mg glucose from starch in 1 h by 1 g ZADO® (pH 4.6 and 40 °C).

‡ One unit (U) is defined as enzyme activity required to release 1 μ mol reducing sugar from 4 mg/ml Na carboxymethyl cellulose in 1 min (pH 5.5 and 37 °C).

§ One unit (U) is defined as enzyme needed to produce 1 μ mol amino acids from protein in 1 min (pH 5.5 and 37 °C).

The flow rate setpoint was adjusted by increments of 0–0.01 ml/min. An aliquot (1 μ l) of the solvent containing the extract of *S. babylonica* was injected into the GC column with the injector heater at 300 °C. The MS was operated in full scan mode (40–650 *m/z* at a rate of two scans per second) with electron impact ionization (EI mode) at 70 electron volts (eV) at an ion source temperature of 230 °C. The relative proportion of constituents was expressed as mg/g of peak area normalization. Identification of extract components was based on direct comparison of the retention times and mass spectral data, computer search matching with the National Institute of Standards and Technology (NIST) MS Search 2.0 library, and by comparison of the fragmentation patterns of mass spectra data with those reported in the literature MS.

Laboratory tests

Samples of feed and faeces were weighed, dried in a forced air oven (60 °C for 48 h) and ground in a Wiley mill (3 mm). Conventional analysis of feed and faecal samples was carried out according to AOAC (1990) for DM (#934.01), ash (#942.05), N (#954.01) and ether extract (EE, #920.39). The neutral detergent fibre (NDF, Van Soest *et al.* 1991), acid detergent fibre (ADF) and lignin (AOAC 1990; #973.18) analyses were conducted using an ANKOM200 Fibre Analyser unit (ANKOM Technology Corporation, Macedon, NY, USA). Neutral detergent fibre was assayed with α -amylase in the NDF. Both NDF and ADF are expressed without residual ash. Urine samples were subjected to determination of N (AOAC 1990) and of PD and creatinine in urine according to the method described by Balcells *et al.* (1992).

Calculations and statistical analysis

Microbial nitrogen (MN) was determined in mmol/day using the following equation (Belenguer *et al.* 2002):

$$\text{MN} = \frac{\text{PD} \div 0.52}{0.92 \times 1.97}$$

where PD, purine derivatives (mmol/day), MN, microbial nitrogen (g/day). Metabolizable protein (MP) was determined by Alderman & Cottrill (1993):

$$\text{MP(gN/day)} = \text{MN} \times 0.75 \times 0.85$$

The growth performance data were analysed with the PROC MIXED procedure of SAS (2002) in which a completely randomized design was used with the statistical model $Y_{ij} = \mu + T_i + E_{ij}$, where Y_{ij} is every observation of the *j*th lamb assigned to *i*th treatment, T_i is the treatment effect, and E_{ij} is the residual error. Comparisons of results were performed using Tukey's test at $P < 0.05$ (Steel & Torrie 1980).

RESULTS

About 59 compounds were identified in *S. babylonica* extract; all of them were C10–C60 compounds: tritriacontane (an aliphatic hydrocarbon: 15.2 mg/g), 9-octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E, a trioleoylglycerol: 11.1 mg/g), hexadecanoic acid-methyl ester (a saturated fatty acid: 10.5 mg/g), 1,3-dioxane-4-(hexadecyloxy)-2-pentadecyl (a heterocyclic organic compound: 10.3 mg/g) and phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol: 9.7 mg/g). There were also some aliphatic hydrocarbons such as nonadecane (1.2 mg/g) and hexatriacontane (0.8 mg/g), and carboxylic acid in the form of oxygenated hydrocarbons (Table 3).

Analysis of enzyme activity showed greater content (U/g) of α -amylase and protease with a reasonable content from cellulase and xylanase (Table 2).

Table 3. *Principal chemical constituents identified in Salix babylonica leaf extracts by GC/MS analysis (adapted from Salem et al. 2011b)*

Compound	Retention time (min)	Chemical formula	MW*	Concentration (mg/g) †
2-hydroxy-6-methyl-benzaldehyde	7.7	C ₈ H ₈ O ₂	136.2	9.9
2-Methoxy-4-vinylphenol	9.8	C ₉ H ₁₀ O ₂	150.0	3.6
Hexatriacontane	13.2	C ₃₆ H ₇₄	507.0	7.7
Nonadecane	14.3	C ₁₉ H ₄₀	268.5	11.7
Tridecanoic acid, 12-methyl, methyl ester	14.5	C ₁₅ H ₃₀ O ₂	242.4	6.7
3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)	15.7	C ₂₀ H ₄₀ O	296.5	97.2
Hexadecanoic acid, methyl ester (palmitic acid, methyl ester)	16.5	C ₁₇ H ₃₄ O ₂	270.5	149.7
9-octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	18.0	C ₅₇ H ₁₀₄ O ₆	885.4	110.5
Octadecanoic acid, methyl ester	18.2	C ₁₉ H ₃₆ O ₂	296.5	16.8
1,3-dioxane, 4-(hexadecyloxy)-2-pentadecyl	18.7	C ₃₅ H ₄₉ O ₃	517.0	103.3
Tritetracontane	19.5	C ₄₃ H ₈₈	605.2	152.1
1-pentacontanol	21.6	C ₅₀ H ₁₀₂ O	719.3	9.7

* MW, molecular weight of the compound (g/mol).

† Concentration based on the total areas of the identified peaks.

Initial metabolic weight (LW)^{0.75} of lambs showed homogeneity ($P = 0.988$) between different treatments. Lambs from treatment EZSB had the greatest ($P \leq 0.01$) ADG (g/day) throughout the period of the experiment. However, during the first 30 days, treatment SB had greater ($P < 0.05$) ADG *versus* EZ and the control; while the EZ treatment increased ($P < 0.05$) ADG *versus* SB and the control during the last 30 days of the experiment. The FC ratios of lambs from the three experimental treatments increased *versus* the control in the order EZSB followed by EZ and then SB ($P < 0.001$) (Table 4).

Water consumption increased most ($P = 0.001$) in SB, followed by EZ during the first 15 days and in EZSB during the second 15 days (i.e. after 30 days) compared to the control. However, during the period from 45 to 60 days, lambs from EZSB consumed more water ($P < 0.05$) (Table 4).

Intake of DM and organic matter (OM) increased ($P < 0.05$) in EZSB followed by EZ treatments compared to the other treatments, whereas NDF and ADF intakes were greater ($P < 0.001$) in EZ treatments. Regarding nutrient digestibility, EZSB increased ($P \leq 0.001$) DM and OM digestibilities. However, the SB treatment increased ($P = 0.001$) ADF digestibility compared to other treatments (Table 4).

The greatest N intake ($P = 0.002$) was observed with EZ treatments, with less N excreted in the urine

($P = 0.001$): the control group had lower N intake but the greatest ($P = 0.001$) N extraction in faeces. The greatest value for N balance ($P = 0.001$) was recorded with the EZSB treatment followed by EZ ($P = 0.001$) (Table 5).

Concentrations of allantoin, total PD, allantoin: creatinine ratio ($P = 0.002$), and PD:creatinine ($P = 0.001$) were increased in EZSB compared to the other treatments, while uric acid concentration increased ($P = 0.001$) in EZ. Both MN (g N/day; $P = 0.001$) and MP (g N/day; $P = 0.005$) increased most in EZSB, followed by the control, compared to the other treatments (Table 5).

DISCUSSION

Daily weight gain, nutrient intake and digestibility

A limited number of exogenous enzymes and phyto-genic extracts have been commercially introduced onto the market. In the present study, the ADG of lambs throughout the experimental period (i.e. 60 days) was improved by 51.6% with EZSB followed by 13.3% for EZ, and then by 8.2% for SB compared with the control treatment. The different feed additives (i.e. EZ, SB, EZ + SB) improved the intake and digestibility of DM, OM, NDF and ADF compared to the control. These improvements were reflected in the

Table 4. Average daily weight gain (g/day), water consumption (l/day), feed intake (g/day) and digestibility (g digested/g ingested) of the diets supplemented with exogenous enzymes and/or *Salix babylonica* extract in lambs ($n = 5$)

	Diets*				S.E.M.	P value
	Control	EZ	SB	EZSB		
Initial live weight (g/kg ^{0.75})	13	12	13	12	2.5	0.988
Average daily weight gain after						
Day 15	96	105	116	150	15.4	0.002
Day 30	121	119	130	162	16.8	0.011
Day 45	101	121	102	157	12.6	<0.001
Day 60	97	125	101	160	13.1	<0.001
Feed conversion	5.9	5.1	5.7	4.4	0.87	<0.001
Water intake after						
Day 15	0.90	0.93	1.42	0.91	0.322	0.001
Day 30	0.86	0.96	1.25	0.97	0.715	0.002
Day 45	1.43	1.39	1.36	1.58	0.912	0.001
Day 60	1.55	1.59	1.54	1.65	0.187	0.032
Intake and digestibility						
Dry matter						
Intake	567	636	574	699	21.4	<0.001
Digestibility	0.703	0.857	0.777	0.877	0.0582	<0.001
Organic matter						
Intake	521	578	528	685	15.9	0.01
Digestibility	0.746	0.878	0.810	0.889	0.0316	0.001
Crude protein						
Intake	412	465	437	452	14.3	0.002
Digestibility	0.721	0.724	0.727	0.722	0.0245	0.001
Neutral detergent fibre						
Intake	160	228	186	201	13.6	0.001
Digestibility	0.601	0.665	0.676	0.694	0.1625	0.001
Acid detergent fibre						
Intake	108	154	125	135	10.4	<0.001
Digestibility	0.455	0.539	0.592	0.561	0.0943	0.001

* Diets supplemented with of exogenous enzyme (EZ, 10 g/h/day), *S. babylonica* (SB, 30 ml/h/day) extract and their combination (EZSB).

feed conversion ratios, which improved by 25.7% for EZSB, 13.5% for EZ and 3.2% for SB treatments. The increased feed intake, nutrient digestibility and daily gain with the administration of SB extract and/or enzymes may be related to the positive effects of metabolites contained in the extract (Jiménez-Peralta *et al.* 2011; Rivero *et al.* 2012; Salem *et al.* 2014a, b), and/or the action exerted by the enzymes (Rivero *et al.* 2012). Researchers have demonstrated that a positive correlation between feeding animals diets supplemented with fibre-degrading enzymes, their performance and modes of action are uncertain (Alsersy *et al.* in press). Hydrolysis of dietary fibre before ingestion, provision of readily fermentable

substrates for ruminal micro-organisms and enhanced synergism in the activity between microbial enzymes in the rumen are possible mechanisms (Morgavi *et al.* 2004; Holtshausen *et al.* 2011; Salem *et al.* 2013). Moreover, other positive improvements were observed as a result of administering enzymes, including enhanced microbial colonization of feed by increasing numbers of ruminal fibrolytic microbes (Morgavi *et al.* 2000) or non-fibrolytic microbes (Colombatto *et al.* 2003), increased rate of fibre degradation in the rumen, increased rumen MP synthesis (Salem *et al.* 2013, 2014a; Alsersy *et al.* in press) and total tract digestibility (Gado *et al.* 2011; Khattab *et al.* 2011). However, the increased digestion

Table 5. Nitrogen utilization, purine derivatives and creatinine excretion in lambs fed maize silage and concentrate supplemented with mixture of exogenous enzymes and/or *Salix babylonica* extract ($n = 5$)

	Diets*				S.E.M.	P value
	Control	EZ	SB	EZSB		
N utilization (g/day)						
N intake	33	47	38	41	4.1	0.002
N excreted						
faeces	5.7	5.1	4.8	4.1	0.54	0.001
Urine	12	21	17	12	1.2	0.001
N balance	16	21	16	26	2.3	0.001
Purine derivatives (PD), mmol/day						
Allantoin (A)	12	13	13	14	1.0	0.001
Uric acid	4	5	5	4	1.8	0.001
Total PD	22	18	16	24	1.4	0.001
Creatinine (C)	26	18	18	17	1.4	0.052
Ratio						
A/C	0.50	0.75	0.69	0.86	0.053	0.002
PD/DOMI [†] (mmol/kg)	0.027	0.016	0.018	0.024	0.0092	0.057
PD/C (mmol/mmol)	0.83	1.03	0.90	1.43	0.043	0.001
MN (gN/day) [‡]	23	19	17	25	1.5	0.001
MP (gN/day) [§]	10	8	8	11	1.0	0.005

* Diets supplemented with of exogenous enzyme (EZ, 10 g/h/day), *S. babylonica* (SB, 30 ml/h/day) extract and their combination (EZSB).

† DOMI, dry organic matter intake.

‡ MN, Microbial nitrogen = (PD/0.52)/(0.92 × 1.97) = X mmol/day.

§ MP, Metabolizable protein (gN/day) = MN × 0.75 × 0.85 (Alderman & Cottrill 1993).

of fibre fractions in the EZ treatment compared to the control may also be related to reduced digesta viscosity (Hristov *et al.* 2000) or altered ruminal fermentation (Nsereko *et al.* 2002). Previous reports using the same enzyme product have also shown that nutrient digestibility was increased (Gado *et al.* 2011; Khattab *et al.* 2011; Salem *et al.* 2013).

Although about 59 compounds were identified in *S. babylonica*, none of the individual active compounds was tested for its effect on animal nutrition. In contrast, many studies with whole crude SB extract (Salem *et al.* 2011a, 2014a) showed improved animal growth performance and nutrient digestion when the extract was administered to animals. These effects may be due to positive impacts of plant secondary metabolites on ruminal micro-organism activity (Jiménez-Peralta *et al.* 2011; Salem *et al.* 2012). Administration of plant extracts may increase muscle deposition, with improved meat quality (Mapiye *et al.* 2010), due to increased amino acid flow to the duodenum (Mueller-Harvey 2006). Improved daily weight gain may also be related to the ability of SB extract to reduce the amount of methane produced

during fermentation, making more energy available for growth (Jiménez-Peralta *et al.* 2011). In fact, about 80–120 g/kg of the digestible energy ingested by ruminants is lost in the rumen as methane (Busquet *et al.* 2006). Within the rumen, some bacterial species are capable of metabolizing the active compounds of the extract, including phenolics (Varel *et al.* 1991), alkaloids (Wachenheim *et al.* 1992) and saponins (Hu *et al.* 2005; Hart *et al.* 2008), and utilize them as an energy source. These metabolites may also act as catalysts for fibre degradation through increasing access of fibrolytic bacteria to the cell-wall components (Jiménez-Peralta *et al.* 2011). Phenylpropanoic acid and phenylacetic acid have been reported to improve cellulose degradation and growth of *Ruminococcus albus* (Stack & Cotta 1986).

The increased water consumption in the SB treatment may be due to the astringency of the extract. Mera Álvarez (2004) found that the tannins present in the plants can precipitate the salivary proteins, causing an unpleasant astringent taste in the mouth. Forbes (1995) reported that animals consume more

water to adjust the osmotic balance in the gastrointestinal tract.

Nitrogen utilization and purine derivatives

Both the EZ and EZSB treatments increased N intake, by about 42.1 and 25.5%, respectively, followed by SB treatments with 15.8%, compared to the control. At the same time, EZSB and EZ treatments improved N balance by 64.5 and 36.8% compared to the control. However, plant extracts rich with active metabolites such as tannins can improve N utilization by impacting and binding with plant proteins in the rumen and preventing microbial degradation, causing an increased amino acid flow to the duodenum from the rumen (Mueller-Harvey 2006). Besides having a protective effect on protein in the rumen, SB extract has the ability to promote duodenal absorption of amino acids and minimize the excretion of N (Jiménez-Peralta *et al.* 2011; Athanasiadou & Kyriazakis 2004). Salem *et al.* (2014a) stated that *S. babylonica* extract supplementation to the diet of growing lambs caused a greater ADG with increased DM intake during the experimental period.

Increased N intake with both EZ and EZSB treatments may be related to increased nutrient digestibility caused by the addition of enzymes (Gado *et al.* 2009; Salem *et al.* 2013; Alsersy *et al.* in press), which may be due to creation of a stable enzyme–feed complex (Kung *et al.* 2000; Colombatto *et al.* 2003). The current results indicate a better utilization of nutrients, reducing environmental pollution in the soil and the amount of excess N excreted, therefore avoiding transformation of N to the greenhouse gas nitrous oxide (N₂O) (Elizondo Salazar 2006). The current results are similar to Pinos-Rodríguez *et al.* (2002), who indicated that fibrolytic enzymes have a favourable impact on N balance in sheep fed alfalfa or ryegrass hay.

Some authors have established a relationship between the amount of digestible OM intake and the urinary excretion of PD (Balcells *et al.* 1993; Pérez *et al.* 1998). However, others found no differences in PD excretion in urine with different intakes of OM (Chen *et al.* 1992). Positive relationships have been described between DM intake and urinary excretion of PD (Vercoe 1976) or OM digestibility and urinary excretion of PD in sheep (Balcells *et al.* 1993), goat (Lindberg 1985) and cow (Liang *et al.* 1994). The values reported in the present study for EZSB are greater than those reported for sheep (18.9–22.3)

(Antoniewicz & Pisulewski 1982; Balcells *et al.* 1993). Salem *et al.* (2013) found that feeding steers on ZADO[®] caused an increase in total PD and allantoin compared to those of the control treatment (i.e. no enzyme addition). However, Gado *et al.* (2009) found no differences between Brown Swiss cows fed the same enzyme preparation (i.e. ZADO[®]) and those fed no enzymes.

The efficiency of MP synthesis increases with increasing feed intake (ARC 1984). The current results indicate that when lambs' diets were supplemented with SB extract and enzymes, the quantity of MP available for metabolism and the net energy density of the ZADO[®] enzyme diet increased. Addition of SB extract was expected to have a positive effect on increasing degradabilities of CP and cell-wall constituents, as well as increasing MP; however, in the present study it had no effect on protein digestibility, with only numerically increased NDF digestibility. This may be related to the concentrations of active compounds and metabolites in the extract (Salem *et al.* 2014a, b). Salem *et al.* (2014a) found that administration of SB extract caused an *in vitro* ruminal MP production from lambs fed increased concentrate diet.

From the current results, it was observed that cumulative positive effects were obtained as a result of combining SB extract with enzymes. The most important factor should be the dose of the additives administered (Jiménez-Peralta *et al.* 2011; Salem *et al.* 2013). Rivero *et al.* (2012, 2013) stated positive effects on Suffolk lamb performance with no effects on blood chemistry when both SB extract and ZADO[®] enzymes were administered together.

CONCLUSION

The supplementation of *S. babylonica* extract (30 ml/day) with exogenous enzymes ZADO[®] (10 g/day), given as an additive in a basal diet of growing lambs, improved their DM and OM digestibility and their live weight. Combination of the two feed additives had a positive impact on the use of N and MP synthesis.

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REFERENCES

- ALDERMAN, G. & COTTRILL, B. R. (1993). *Energy and Protein Requirements of Ruminants. An Advisory Manual Prepared by the AFRC Technical Committee on Responses to Nutrients*. Wallingford, UK: CAB International.
- ALSERSY, H., SALEM, A. Z. M., BORHAMI, B. E., OLIVARES, J., GADO, H. M., MARIEZCURRENA, M. D., YACUOT, M. H., KHOLIF, A. E., EL-ADAWY, M. & HERNANDEZ, S. R. (in press). Effect of Mediterranean saltbush (*Atriplex halimus*) ensilaging with two developed enzyme cocktails on feed intake, nutrient digestibility and ruminal fermentation in sheep. *Animal Science Journal*. doi: 10.1111/asj.12247.
- ANTONIEWICZ, A. M. & PISULEWSKI, P. M. (1982). Measurement of endogenous allantoin excretion in sheep urine. *Journal of Agricultural Science, Cambridge* **98**, 221–223.
- AOAC (1990). *Official Methods of Analysis of the Association of Official Analytical Chemists*. Vol. 1. 15th Ed. Washington, DC: Association of Official Analytical Chemists.
- ARC (1984). *The Nutrient Requirement of Ruminant Livestock*. Farnham Royal, Slough, UK: Commonwealth Agricultural Bureaux.
- ATHANASIADOU, S. & KYRIAZAKIS, I. (2004). Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. *Proceedings of the Nutrition Society* **63**, 631–639.
- BALCELLS, J., GUADA, J. A., PEIRO, J. M. & PARKER, D. S. (1992). Simultaneous determination of allantoin and oxipurines in biological fluids by high performance liquid chromatography. *Journal of Chromatography* **575**, 153–157.
- BALCELLS, J., FONDEVILA, M., GUADA, J. A., CASTRILLO, C. & SURRA, J. C. E. (1993). Urinary excretions of purine derivatives and nitrogen in sheep given straw supplemented with different sources of carbohydrates. *Animal Production* **57**, 287–292.
- BELONGUER, A., YAÑEZ, D., BALCELLS, J., OZDEMIR-BABER, N. H. & GONZALEZ-RONQUILLO, M. (2002). Urinary excretion of purine derivatives and prediction of rumen microbial outflow in goats. *Livestock Science* **77**, 127–135.
- BERNFELD, P. (1955). Amylases, alpha and beta. *Methods in Enzymology* **1**, 149–158.
- BUSQUET, M., CALSAMIGLIA, S., FERRET, A. & KAMEL, C. (2006). Plant extracts affect in vitro rumen microbial fermentation. *Journal of Dairy Science* **89**, 761–771.
- CHEN, X. B., CHEN, Y. K., FRANKLIN, M. F., ORSKOV, E. R. & SHAND, W. J. (1992). The effect of feed intake and body weight on purine derivate excretion and microbial protein supply in sheep. *Journal of Animal Science* **70**, 1534–1542.
- COLOMBATTO, D., MOULD, F. L., BHATT, M. K., MORGAVI, D. P., BEAUCHEMIN, K. A. & OWEN, E. (2003). Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms in vitro. *Journal of Animal Science* **81**, 1040–1050.
- DURMIC, Z. & BLACHE, D. (2012). Bioactive plants and plant products: effects on animal function, health and welfare. *Animal Feed Science and Technology* **176**, 150–162.
- ELIZONDO SALAZAR, J. (2006). El nitrógeno en los sistemas ganaderos de leche. *Agronomía Mesoamericana* **17**, 69–77.
- FORBES, J. M. (1995). *Voluntary Food Intake and Diet Selection in Farm Animals*. Wallingford, UK: CAB International.
- GADO, H. M., SALEM, A. Z. M., ROBINSON, P. H. & HASSAN, M. (2009). Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Animal Feed Science and Technology* **154**, 36–46.
- GADO, H. M., SALEM, A. Z. M., ODONGO, N. E. & BORHAMI, B. E. (2011). Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology* **165**, 131–136.
- HART, K. J., YAÑEZ-RUIZ, D. R., DUVAL, S. M., MCEWAN, N. R. & NEWBOLD, C. J. (2008). Plant extracts to manipulate rumen fermentation. *Animal Feed Science and Technology* **147**, 8–35.
- HOLTSHAUSEN, L., CHUNG, Y.-H., GERARDO-CUERVO, H., OBA, M. & BEAUCHEMIN, K. A. (2011). Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. *Journal of Dairy Science* **94**, 899–907.
- HRISTOV, A. N., MCALLISTER, T. A. & CHENG, K.-J. (2000). Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: effects on nutrient digestion in cattle fed a barley grain diet. *Journal of Animal Science* **78**, 477–487.
- HU, W.-L., LIU, J.-X., YE, J.-A., WU, Y.-M. & GUO, Y.-Q. (2005). Effect of tea saponin on rumen fermentation in vitro. *Animal Feed Science and Technology* **120**, 333–339.
- JIMÉNEZ-PERALTA, F. S., SALEM, A. Z. M., MEJÍA-HERNÁNDEZ, P., GONZÁLEZ-RONQUILLO, M., ALBARRÁN-PORTILLO, B., ROJO-RUBIO, R. & TINOCO-JARAMILLO, J. L. (2011). Influence of individual and mixed extracts of two tree species on in vitro gas production kinetics of a high concentrate diet fed to growing lambs. *Livestock Science* **136**, 192–200.
- KAMEL, C. (2001). Tracing modes of action and roles of plant extracts in non-ruminants. In *Recent Advances in Animal Nutrition* (Eds P. C. Garnsworthy & J. Wiseman), pp. 135–150. Nottingham, UK: Nottingham University Press.
- KHATTAB, H. M., GADO, H. M., KHOLIF, A. E., MANSOUR, A. M. & KHOLIF, A. M. (2011). The potential of feeding goats sun dried rumen contents with or without bacterial inoculums as replacement for berseem clover and the effects on milk production and animal health. *International Journal of Dairy Science* **6**, 267–277.
- KUNG, L., JR., TREACHER, R. J., NAUMAN, G. A., SMAGALA, A. M., ENDRES, K. M. & COHEN, M. A. (2000). The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science* **83**, 115–122.
- LANCASHIRE, P. D., BLEIHOLDER, H., LANGELÜDDECKE, P., STAUSS, R., VAN DEN BOOM, T., WEBER, E., & WITZENBERGER, A. (1991). A uniform decimal code for growth stages of crops and weeds. *Annals of Applied Biology* **119**, 561–601.

- LIANG, J. B., MATSUMOTO, M. & YOUNG, B. A. (1994). Purine derivative excretion and ruminal microbial yield in Malaysian cattle and swamp buffalo. *Animal Feed Science and Technology* **47**, 189–199.
- LIN, Y., GARY, E. & ROBERT, E. (1969). Action of proteolytic enzymes on N,N-dimethyl proteins. Basis for a micro-assay for proteolytic enzymes. *Journal of Biological Chemistry* **244**, 789–793.
- LINDBERG, J. E. (1985). Urinary allantoin excretion and digestible organic matter intake in dairy goats. *Swedish Journal of Agricultural Research* **15**, 31–37.
- MAPIYE, C., CHIMONYO, M., DZAMA, K., STRYDOM, P. E. & MUCHENJE, V. (2010). Meat quality attributes of Nguni steers supplemented with *Acacia karroo* leaf-meal. *Meat Science* **8**, 621–627.
- MCGUFFEY, R. K., RICHARDSON, L. F. & WILKINSON, J. I. D. (2001). Ionophores for dairy cattle: current status and future outlook. *Journal of Dairy Science* **84**, E194–E203.
- MEJÍA-HERNÁNDEZ, P., SALEM, A. Z. M., ELGHANDOUR, M. M. Y., CIPRIANO-SALAZARA, M., CRUZ-LAGUNAS, B. & CAMACHO, L. M. (2014). Anthelmintic effects of *Salix babylonica* L. and *Leucaena leucocephala* Lam. extracts in growing lambs. *Tropical Animal Health and Production* **46**, 173–178.
- MERA ÁLVARES, M. I. (2004). *Efecto de Leguminosas Forrajeras Tropicales Ricas en Taninos Sobre la Fermentación Ruminal y la Producción de Metano en un Sistema in Vitro (RUSITEC)*. Palmira, Colombia: CIAT.
- MORGAVI, D. P., NSEREKO, V. L., RODE, L. M., BEAUCHEMIN, K. A., MCALLISTER, M. & WANG, Y. (2000). A *Trichoderma* feed enzyme preparation enhances adhesion of *Fibrobacter succinogenes* to complex substrates but not to pure cellulose. In Proceedings of the 25th Conference on Rumen Function, Chicago, Illinois, US.
- MORGAVI, D. P., BEAUCHEMIN, K. A., NSEREKO, V. L., RODE, L. M., MCALLISTER, T. A., IWAASA, A. D., WANG, Y. & YANG, W. Z. (2001). Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. *Journal of Animal Science* **79**, 1621–1630.
- MORGAVI, D. P., BEAUCHEMIN, K. A., NSEREKO, V. L., RODE, L. M., MCALLISTER, T. A. & WANG, Y. (2004). *Trichoderma* enzymes promote *Fibrobacter succinogenes* S85 adhesion to, and degradation of, complex substrates but not pure cellulose. *Journal of the Science of Food and Agriculture* **84**, 1083–1090.
- MUELLER-HARVEY, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* **86**, 2010–2037.
- NSEREKO, V. L., BEAUCHEMIN, K. A., MORGAVI, D. P., RODE, L. M., FURTADO, A. F., MCALLISTER, T. A., IWAASA, A. D., YANG, W. Z. & WANG, Y. (2002). Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Canadian Journal of Microbiology* **48**, 14–20.
- Official Journal of The European Union (2003). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union* **L268**, 29–43.
- PALMA, J. M., DELGADO, C., RODRÍGUEZ, A. & AGUIRRE, M. A. (1995). *Composición Química y Digestibilidad de tres Leguminosas Arbóreas. Memorias primer simposio Estatal de ciencia y Tecnología*. Colima, México: Universidad de Colima.
- PÉREZ, J. F., BALCELLS, J., CEBRIAN, J. A. & MARTÍN-ORUE, S. M. (1998). Excretion of endogenous and exogenous purine derivatives in sheep: effect of increased concentrate intake. *British Journal of Nutrition* **79**, 237–240.
- PINOS-RODRÍGUEZ, J. M., GONZÁLEZ, S. S., MENDOZA, G. D., BÁRCENA, R., COBOS, M. A., HERNÁNDEZ, A. & ORTEGA, M. E. (2002). Effect of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and ryegrass hay fed to lambs. *Journal of Animal Science* **80**, 3016–3020.
- RIVERO, N., SALEM, A. Z. M., GADO, H. M., GONZÁLEZ-RONQUILLO, M., PLIEGO, A. B., PEÑUELAS, C. G. & ODONGO, N. E. (2012). Effect of exogenous enzymes and *Salix babylonica* extract or their combination on haematological parameters in growing lambs. *Journal of Animal and Feed Sciences* **21**, 577–586.
- RIVERO, N., SALEM, A. Z. M., RONQUILLO, M. G., CERRILLO-SOTO, M. A., CAMACHO, L. M., GADO, H. & PEÑUELAS, C. G. (2013). Effects of exogenous enzymes and *Salix babylonica* L. extract on cellular immune response and its correlation with average daily weight gain in growing lambs. *Animal Nutrition and Feed Technology* **13**, 411–422.
- ROBYT, J. F. & WHELAN, W. J. (1972). Reducing value methods for maltodextrins: 1. Chain-length dependence of alkaline 3,5-dinitrosalicylate and chain length independence of alkaline copper. *Analytical Biochemistry* **45**, 510–516.
- RUSSELL, J. B. & HOULIHAN, A. J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiology Reviews* **27**, 65–74.
- SALEM, A. Z. M. (2012). Oral administration of leaf extracts to rumen liquid donor lambs modifies in vitro gas production of other tree leaves. *Animal Feed Science and Technology* **176**, 94–101.
- SALEM, A. Z. M., OLIVARES, M., LOPEZ, S., GONZALEZ-RONQUILLO, M., ROJO, R., CAMACHO, L. M., CERRILLO, S. M. A. & MEJIA, H. P. (2011a). Effect of natural extracts of *Salix babylonica* and *Leucaena leucocephala* on nutrient digestibility and growth performance of lambs. *Animal Feed Science and Technology* **170**, 27–34.
- SALEM, A. Z. M., SALEM, M. Z. M., GONZALEZ-RONQUILLO, M., CAMACHO, L. M. & CIPRIANO, M. (2011b). Major chemical constituents of *Leucaena leucocephala* and *Salix babylonica* leaf extracts. *Journal of Tropical Agriculture* **49**, 95–98.
- SALEM, A. Z. M., HASSAN, A. A., KHALIL, M. S., GADO, H. M., ALSERSY, H. & SIMBAYA, J. (2012). Effects of sun-drying and exogenous enzymes on nutrients intake, digestibility and nitrogen utilization in sheep fed *Atriplex halimus* foliages. *Animal Feed Science and Technology* **171**, 128–135.
- SALEM, A. Z. M., GADO, H. M., COLOMBATTO, D. & ELGHANDOUR, M. M. Y. (2013). Effects of exogenous

- enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. *Livestock Science* **154**, 69–73.
- SALEM, A. Z. M., KHOLIF, A. E., OLIVARES, M., ELGHANDOUR, M. M. Y., MELLADO, M. & ARECE, J. (2014a). Influence of *S. babylonica* extract on feed intake, growth performance and diet in vitro gas production profile in young lambs. *Tropical Animal Health and Production* **46**, 213–219.
- SALEM, A. Z. M., KHOLIF, A. E., ELGHANDOUR, M. M. Y., BUENDÍA, G., MARIEZCURRENA, M. D., HERNANDEZ, S. R. & CAMACHO, L. M. (2014b). Influence of oral administration of *Salix babylonica* extract on milk production and composition in dairy cows. *Italian Journal of Animal Science* **13**, 10–14.
- SALEM, A. Z. M., KHOLIF, A. E., ELGHANDOUR, M. M. Y., HERNANDEZ, S. R., DOMÍNGUEZ-VARA, I. A. & MELLADO, M. (2014c). Effect of increasing levels of seven tree species extracts added to a high concentrate diet on in vitro rumen gas output. *Animal Science Journal*. doi: 10.1111/asj.12218.
- SAS Institute (2002). *SAS User's Guide: Statistics*. Version 9.0. Cary, NC, USA: SAS Institute.
- STACK, R. J. & COTTA, M. A. (1986). Effect of 3-phenylpropanoic acid on growth of, and cellulose utilization by, cellulolytic ruminal bacteria. *Applied Environmental Microbiology* **52**, 209–210.
- STEEL, R. G. D. & TORRIE, J. H. (1980). *Principles and Procedures of Statistics: A Biometrical Approach*. New York: McGraw-Hill Book Co.
- VAN SOEST, P. J., ROBERTSON, J. B. & LEWIS, B. A. (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**, 3583–3597.
- VAREL, V. H., JUNG, H. G. & KRUMHOLZ, L. R. (1991). Degradation of cellulose and forage fiber fractions by ruminal cellulolytic bacteria alone and in coculture with phenolic monomer-degrading bacteria. *Journal of Animal Science* **69**, 4993–5000.
- VASTA, V. & LUCIANO, G. (2011). The effects of dietary consumption of plants secondary compounds on small ruminants' products quality. *Small Ruminant Research* **101**, 150–159.
- VERCOE, J. E. (1976). Urinary allantoin excretion and digestible dry matter intake in cattle and buffalo. *Journal of Agriculture Science, Cambridge* **86**, 613–615.
- WACHENHEIM, D. E., BLYTHE, L. L. & CRAIG, A. M. (1992). Characterization of rumen bacterial pyrrolizidine alkaloid biotransformation in ruminants of various species. *Veterinary and Human Toxicology* **34**, 513–517.
- WALLACE, R. J., MCEWAN, N. R., MCINTOSH, F. M., TEFEREDEGNE, B. & NEWBOLD, C. J. (2002). Natural products as manipulators of rumen fermentation. *Asian-Australasian Journal of Animal Sciences* **15**, 1458–1468.