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Toxicity of endophyte-infected ryegrass hay containing high ergovaline level in lactating ewes[1]

N. Zbib,* C. Repussard,* D. Tardieu,* N. Priymenko,† C. Domange,† and P. Guerre*[2]

*Université de Toulouse, INP, ENVT, UR Mycotoxicologie, F-31076 Toulouse, France;
and †Université de Toulouse, INP, ENVT, INRA UMR1331 Toxalim, F-31076 Toulouse, France

ABSTRACT: The symbiotic association of *Epichloë festucae* var. *lolii* (formerly named *Neotyphodium lolii*) with perennial ryegrass (*Lolium perenne*) leads to the production of ergovaline (EV) and lolitrem B (LB) that are toxic for livestock. The objectives of this study were to determine the effects of feeding endophyte-infected ryegrass (SE+) hay on 16 lactating ewes (BW 80 ± 10 kg) in comparison with endophyte-free ryegrass (SE–) hay to investigate the putative mechanisms of action of EV and LB and to evaluate their persistence in milk and animal tissues. The mean EV and LB concentrations in SE+ hay were 851 and 884 μg/kg DM, respectively, whereas these alkaloids were below the limit of detection in SE– hay. No effect of SE+ was observed on animal health and skin temperature whereas prolactin decreased and significant differences between hays were observed from d 7 to 28 of the study ($P < 0.03$) but had no effect on milk production. Hematocrit and biochemical analyses of plasma revealed no significant difference between SE+ and SE–, whereas cortisol concentration differed significantly on d 28 ($P = 0.001$). Measurement of oxidative damage and antioxidant enzyme activities in plasma, liver, and kidneys revealed a slight increase in some enzyme activities involved in defense against oxidative damage in the SE+ fed ewes. Slight variations in the activities of hepatic and kidney flavin monooxygenase enzymes were observed, whereas in the kidney, glutathione *S*-transferase activity decreased significantly ($P = 0.002$) in the SE+ fed ewes, whereas uridine diphosphate glucuronosyltransferase activity increased ($P = 0.001$). After 28 d of exposure of ewes to the SE+ hay, low EV and LB concentrations were measured in tissues. The highest concentration of EV was observed in the liver (0.68 μg/kg) whereas fat contained the highest concentration of LB (2.39 μg/kg). Both toxins were also identified at the trace level in milk.

Key words: drug metabolizing enzymes, endophyte-infected perennial ryegrass, ergovaline, ewes, lolitrem B, prolactin.

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INTRODUCTION

The development of *Epichloë festucae* var. *lolii* (formerly named *Neotyphodium lolii*; Leuchtmann et al., 2014) in perennial ryegrass (*Lolium perenne*) leads to the production of several alkaloids belonging to the indole-diterpene and ergopeptine groups (Repussard et al., 2013). Endophyte-infected ryegrass (**SE+**) is responsible for "staggers" in livestock, which is often reported in New Zealand and Australia (Cunningham and Hartley, 1959; Rattray, 2003). Lolitrem B (**LB**), an alkaloid of the indole-diterpene group known to produce tremorgenic symptoms, is recognized as the main alkaloid responsible for staggers in livestock, with a toxic threshold of 1,800 to 2,000 μg/kg DM (Bacon, 1995; Tor-Agbidye et al., 2001; Zbib et al., 2014a). Ergovaline (**EV**), an ergopeptine alkaloid also produced by SE+, is known for its vasomotor properties (Repussard et al., 2013). This compound

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[2]Corresponding author: p.guerre@envt.fr

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is responsible for fescue foot, summer syndrome, and economic loss in livestock after consumption of endophyte-infected tall fescue (**FE+**) with a toxic threshold of 500 to 800 µg/kg DM for sheep (Bacon, 1995; Tor-Agbidye et al., 2001; Zbib et al., 2014a).

Despite the high level of SE+ in Europe, ryegrass staggers is rarely encountered (Bony et al., 1998, 2001; Benkhelil et al., 2004). Monitoring of LB and EV concentrations in SE+ cultivar Samson sown in southern France over a period of 3 yr revealed that the maturity of the plant strongly influenced alkaloids concentration (Repussard et al., 2014c). Interestingly, the highest LB level in SE+ was observed at the fully ripe stage and was lower than the toxic threshold of 2,000 µg/kg DM in sheep, in agreement with previous studies conducted in Germany (Oldenburg, 1997). By contrast, although the highest EV levels in plant was also observed at the fully mature stage, in agreement with what was reported in FE+, the EV levels in SE+ at the fully mature stage was always higher than the toxic threshold of 500 µg/kg DM in sheep reported in FE+ (Repussard et al., 2014b; Tor-Agbidye et al., 2001).

Because the profile of alkaloids' production in plants has a strong influence on their toxicity in livestock (Guerre, 2015; Fletcher, 2013; Gadberry et al., 2003), it appeared of interest to evaluate the toxicity of an SE+ hay that contained high EV and low LB levels on lactating ewes. This study focuses on animal health, milk production, prolactin (**PRL**), skin temperature, oxidative damage, and drug metabolizing enzyme activities that have been reported as targets of EV toxicity in lactating sheep (Zbib et al., 2014b). The risk of persistence of EV and LB in milk and tissue destined for human consumption was investigated.

MATERIALS AND METHODS

Hay Production and Animal Exposure

All experimental procedures involving animals were in accordance with the French national guidelines for the care and use of animals for research purposes (Anonymous, 1998). Perennial ryegrass cv. Grasslands Samson were sown in 2009 at the La Cazotte agricultural school (Saint-Affrique, France) in 2 homogenous plots, one with endophyte-free seeds and the other with standard wild-type endophyte-infected seeds as previously described (Repussard et al., 2014c). In June 2011, hay was harvested and then stored in a hangar until November 2011, when the study on ewes began. An assay with lactating ewes (Lacaune breed) was conducted in a large barn at low stocking density under natural light. From lambing to the beginning of the assay with perennial ryegrass, animals were fed with long-stem Italian ryegrass offered ad libitum on racks self-service. Daily, a complementary feed of 700 g of high protein concentrate (Evialis France, Vedène, France), 500 g of barley, and 20 g of multivitamin supplement (Unicor, Rodez, France) was individually supplied at head locks. Three days before the beginning of the assay, 16 ewes (80 ± 10 kg BW and 35 ± 4 d in milk) were distributed into 2 homogenous groups of 8 each. Around 20 kg of hay was provided daily on racks self-service with free access for all the animals according to the usual practice in this barn. Forage was given in excess and access was maintained all day to avoid competition between ewes. Daily orts were collected from the pen and weighed before new distribution. The observation of animals revealed that ewes were not in competition with their pen mates for the access to forage, visual examination of orts (around 30% of the hay provided) revealed no difference with the forage provided, and access to treatment was considered independent. On Day 0 of the assay, one group was fed with endophyte-free ryegrass (**SE–**) hay and another with SE+ hay. Each week of the assay, 1 kg of hay was taken from 5 different locations of the bales for chemical analysis. Dry matter was 90%. Crude protein was 82 and 104 g/kg DM, crude fiber was 281 and 303 g/kg DM, and net energy for lactation ($\mathbf{NE_l}$) was 1.32 and 1.19 Mcal/kg DM for SE– and SE+, respectively. Ergotamine, ergocryptine, ergocornine, ergosine, ergometrine, ergocristine, and their -inine forms were determined by HPLC–mass spectrometry (Qualtech SA laboratory, Vandoeuvre, France) and were below the limit of detection (**LD**; 3 µg/kg). Ergovaline and LB were determined as described below.

Animal Monitoring and Sampling

Tremors and any mark of disease (prostration and diarrhea) or abnormal comportment were checked daily. Core body temperatures (Tco), skin temperatures (Ts), and air temperatures (Ta) were measured on Days 0, 3, 7, 14, 21, and 28 as described in Zbib et al. (2014b). The thermocirculation index (**TCI**) was calculated (Burton and Edholm, 1955) according to the following equation: TCI = (Ts – Ta)/(Tco – Ts). Body weight, feed consumption, and milk production were recorded on Days 1, 8, and 22. Fat and protein contents and the number of cells in the milk were analyzed according to the French Ministry of Agriculture for the payment of the milk quality (Laboratoire Interprofessionnel d'Analyses Laitières, Aurillac, France). One 50-mL milk sample per ewe per milking was stored at –20°C for EV and LB analysis. Blood samples were collected by jugular vein puncture in lithium heparinized tubes (Terumo, Leuven, Belgium) 1 h after milking on Days 0, 7, 14, 21, and 28. Ewes were killed by exsanguination after stunning at the slaughterhouse (Abattoir Peyrottes, Saint-Affrique,

France) on Day 28. Carcass, liver, and kidneys were examined to reveal anomalies and then weighed. Fifty grams of liver, kidneys, muscle, and abdominal fat were collected and stored at –80°C until analysis.

Blood Analysis

Five hours after blood collection, hematocrit was determined in a microcapillary tube at 11,000 × *g* for 15 min at room temperature. The rest of blood was centrifuged at 3,000 × *g* for 10 min at 4°C and the plasma was collected. A 500-µL aliquot was immediately deproteinized with metaphosphoric acid (1.25 *M*, vol/vol). The rest of the plasma and the deproteinized samples were stored at –80°C until analysis. Biochemical parameters (sodium, potassium, urea, creatinine, total bilirubin, total protein, cholesterol, triglycerides, lactate dehydrogenase [Enzyme Code {**EC**} 1.1.1.27], aspartate aminotransferase [EC 2.6.1.1], alanine aminotransferase [EC 2.6.1.2], and creatine phosphokinase [EC 2.7.3.2]) were assayed using a clinical chemistry analyzer (Hitachi 717; Hitachi Solutions, Tokyo, Japan) following the manufacturer's recommendations in a medical laboratory (Laboratoire d'analyses médicales, La Salvetat Saint-Gilles, France). Prolactin concentration in plasma was assayed by RIA (Kann, 1971) at the Neuroendocrinology Laboratory (INRA, Nouzilly, France). The method permitted a sensitivity of 2.5 ng/mL (intra- and interassay CV of 7–12% and 10–14%, respectively). Plasma cortisol concentrations were measured with the Cayman cortisol EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations.

Ergovaline Determination

Ergovaline standard was kindly provided by G.E. Rottinghaus (College of Veterinary Medicine, University of Missouri, Columbia, MO). Ergovaline concentration in the hay was determined by HPLC according to the method of Rottinghaus et al. (1991), with slight modifications (Zbib et al., 2014b). Briefly, 1 kg of hay was dried at 60°C for 24 h and ground to 0.5 mm. Five grams of the homogenate were extracted with 100 mL of alkaline chloroform (2 h) and filtered on a phase separator (Whatman PS1; GE Healthcare companies, Buckinghamshire, UK). Ten milliliters of the filtered solution were purified on homemade ergosil silica gel columns (Analtech Inc., Newark, DE) as previously described (Zbib et al., 2014b). Ergovaline was eluted with methanol, and the eluate was evaporated to dryness at 45°C under a stream of nitrogen. The dry residue was dissolved in methanol (500 µL) and 20 µL were injected into the HPLC system. Separation was achieved with acetonitrile (35%) in aqueous ammonium carbonate (200 mg/L, 65%) through a 150- by 4.6-mm, 5-µm, 120-Å Prontosil C18 column (Bischoff Chromatography, Leonberg, Germany). Fluorescence was detected at 420 nm after excitation at 250 nm with a RF-10AXL fluorometer (Shimadzu Corporation, Kyoto, Japan). The resulting chromatograms were monitored by PIC 3 software (ICS, Toulouse, France). Ergovaline retention time was 9.5 min. The percentage of recovery was 83%. The limit of quantitation (**LQ**) was 20 ng/g. Samples with EV levels higher than 500 ng/g DM were diluted before new analysis.

Ergovaline in the milk was determined according to Durix et al. (1999), with slight modifications. Briefly, 5 mL of milk were shaken 10 min with 15 mL of acetone for 10 min and then centrifuged at 3,000 × *g* for 10 min at room temperature. The acetone was evaporated, and the aqueous residue was alkalinized with 20 µL of 30% (vol/vol) ammonia solution and extracted twice with 10 mL of chloroform for 1 h on an orbital shaker. The chloroform extracts were pooled, filtered, and purified as previously described for the hay. Forty microliters of the dry residue dissolved in methanol (200 µL) were injected into the HPLC system. The percentage of recovery was 99%. The LQ was 0.7 ng/mL (Durix et al., 1999).

Ergovaline in the tissues was determined according to Tardieu et al. (2015). Briefly, 3 g of liver, kidneys, or muscle were adjusted to a final volume of 9 mL in phosphate buffer (0.1 *M*, pH 7.6) and homogenized 20 s at 3,000 rpm with an Ultra Turrax (TP 18; IKA, Staufen, Germany). Three milliliters of the suspension were collected and extracted twice with 6 mL of chloroform on an orbital shaker for 1 h. Fat and brain samples were directly extracted with chloroform without homogenization. The chloroform extracts were pooled, filtered, and purified as previously described for the hay. Forty microliters of the dry residue dissolved in methanol (200 µL) were injected into the HPLC system. A percentage of recovery of 91% and a LQ of 0.5 ng/g were observed for EV in liver, kidneys, and muscle (Tardieu et al., 2015). A percentage of recovery of 65% and a LQ of 0.83 ng/g were observed for EV in fat and brain (Tardieu et al., 2015). The LD was estimated at around 0.15 ng/g in all tissues.

Lolitrem B Determination

Lolitrem B standard was purchased from AgResearch Limited Ruakura Research Centre (Hamilton, New Zealand). All reagents and chemicals, HPLC grade, were purchased from Sigma Chemical Co. (St. Louis, MO). Lolitrem B in the hay was determined

after extraction with dichloromethane as previously described (Repussard et al., 2014a). Concentrations in the purified extracts were measured after purification on homemade ergosil columns according to the method of Gallagher et al. (1985), with slight modifications (Repussard et al., 2014a). Lolitrem B in milk and tissues was determined by HPLC according to Gallagher et al. (1985) after hexane extraction and solid-phase extraction(**SPE**) purification according to Miyazaki et al. (2004) with slight modifications. Three grams of tissues were added to 3 mL of phosphate buffer (pH 7.4, 0.1 *M*) and homogenized with an Ultra Turrax (TP 18; IKA). Two milliliters of the suspension were collected and extracted with 12 mL of hexane on an orbital shaker for 1 h. Milk samples (3 mL) were diluted with 3 mL of phosphate buffer (pH 7.4, 0.1 *M*) and then extracted using the same protocol as that described for tissues. Phase separation was conducted by freezing for 5 min at –80°C. The supernatant was collected and the pellet extracted twice with 4 mL of hexane using the same procedure. The hexane extracts were pooled before purification on SPE columns prepared as follows: 1 cm (about 150 mg) of ergosil silica gel (Analtech Inc.) was placed in an empty 5-mL SPE cartridge (SiliCycle, Quebec City, QC, Canada) followed by a biological disk (SiliCycle) and 1 cm of sodium sulfate (about 1,000 mg). The columns were preconditioned with 2 mL of hexane before the hexane extracts were passed. Columns were washed with 5 mL of hexane. Lolitrem B was eluted with 6 mL of dichloromethane/acetonitrile (80:20, vol/vol). Elute was collected and evaporated to dryness with a sample concentrator at 45°C under a stream of nitrogen. The dry residue was dissolved in 100 µL dichloromethane/acetonitrile (80:20, vol/vol). Lolitrem B was separated and detected as previously described for hay (Repussard et al., 2014a).

Tissue Fractions and Assays

All chemicals and reagents were provided by Sigma Chemical Co. Separation of tissue fractions and the assays on plasma, microsomal, and cytosolic fractions were done as previously described (Zbib et al., 2014b). Briefly, tissues (the liver and kidneys) were homogenized in phosphate buffer and then centrifuged at 9,000 x g for 35 min at 4°C to remove nuclei and fragments of tissues. The upper phase was then centrifuged at 105,000 x g for 65 min at 4°C to obtain microsomal and cytosolic fractions that were stored at –80°C until analysis. Proteins were dosed using the Bio-Rad protein assay with BSA as the standard (Bio-Rad Laboratories, Munich, Germany). Malondialdehyde was determined fluorometrically after formation of a pink complex with thiobarbituric acid (Buege and Aust, 1978). Oxidized glutathione (**GSSG**) and total glutathione were determined using an optimized enzymatic recycling method before quantification of glutathione (**GSH**) with Ellman's reagent (Baker et al., 1990) in the presence or absence of 2-vinylpyridine (Griffith, 1980). Superoxide dismutase (**SOD**; EC 1.15.1.1) activity was based on the inhibition of the formation of blue formazan by xanthine-xanthine oxidase (EC 1.17.3.2) in the presence of nitroblue tetrazolium (Sun et al., 1988). Superoxide dismutase activity was calculated using bovine erythrocyte SOD as the standard. Catalase (EC 1.11.1.6) activity was estimated by formation of formaldehyde in the presence of H_2O_2 (Johansson and Håkan Borg, 1988). Glutathione reductase (EC 1.6.4.2) reduces GSSG into GSH in the presence of nicotinamide adenine dinucleotide phosphate (**NADPH**; Carlberg and Mannervik, 1984). Glutathione peroxidase (EC 1.11.1.9) reduces cumene hydroperoxide in the presence of GSH to produce GSSG, which is recycled into GSH by glutathione reductase in the presence of NADPH (Paglia and Valentine, 1967). Both activities were measured separately by monitoring the decrease in NADPH.

Drug metabolizing enzyme activities were measured in microsomal or cytosolic tissue fractions of the liver and kidneys. *N*-demethylation activities were measured in the microsomal fraction of tissues with aminopyrine, benzphetamine, ethylmorphine, erythromycin, and *N*,*N*-dimethylnitrosamine as substrates by the formation of formaldehyde, which was detected by the Nash method (Cochin and Axelrod, 1959). Dealkylation of 7-methoxyresorufin, 7-ethoxyresorufin, and 7-penthoxyresorufin was estimated through resorufin formation in the microsomal fraction of tissues (Lake, 1987). Uridine diphosphate glucuronyltransferase activity with *p*-nitrophenol was measured in the microsomal fraction of tissues by monitoring the formation of para-nitrophenol-glucuronide (Frei, 1970). Using 1-chloro-2-4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates, glutathione *S*-transferase activities were determined in the cytosolic fraction of tissues by monitoring the formation of the respective conjugates (Habig et al., 1974).

Statistical Analyses

Statistical analyses were performed using R software (version 2.11.1; R Foundation for Statistical Computing, Vienna, Austria). Body weight, milk production, temperatures, TCI, biochemistry, and PRL and cortisol concentrations measured on each ewe (repeated measures) were compared using a linear model to assess the effect of ewe, hay, time, and the interaction between hay and time. When a significant effect was obtained ($P < 0.05$), a complementary range test (Wilcoxon) was used for

Table 1. Ergovaline and lolitrem B concentrations in ryegrass hay[1] and effect on BW and milk production in ewes. Values are expressed as mean ± SD (n = 8)

	SE–			SE+		
Parameter	Day 1	Day 8	Day 22	Day 1	Day 8	Day 22
Ergovaline,[2] µg/kg	<5	<5	<5	1,006 ± 397	674 ± 33	872 ± 351
Lolitrem B,[2] µg/kg	<10	<10	<10	1,027 ± 139	824 ± 28	800 ± 301
Feed intake,[3] kg/ewe·d	1.35	1.23	1.52	1.53	1.12	1.46
BW, kg	81 ± 10	79 ± 10	80 ± 10	80 ± 9	77 ± 8	79 ± 9
Milk production, L/d	2.6 ± 0.6	2.3 ± 0.6	1.8 ± 0.6†	2.6 ± 0.5	2.2 ± 0.4	1.8 ± 0.6†
Fat content, g/L	49 ± 10	63 ± 6†	61 ± 10†	50 ± 7	66 ± 5†	63 ± 6†
Protein content, g/L	51 ± 4	47 ± 5	51 ± 4	52 ± 4	49 ± 6	51 ± 6

[1]SE– = endophyte-free ryegrass; SE+ = endophyte-infected ryegrass.

[2]Mean ± SD concentration measured at the pen level for 1 kg of hay taken from 5 different locations of the bales.

[3]Mean consumption calculated by dividing the consumption measured at the pen level by the number of ewes (n = 8).

†Significant difference from Day 1 (Wilcoxon, $P < 0.05$).

the individual comparison of means. No effect of ewe was observed whatever the variable analyzed. Oxidative stress parameters and biotransformation enzyme activities were compared using Student's t test after testing for normality (Shapiro) and equality of variance (Fisher). Significantly different groups ($P < 0.05$) are identified by a superscript. Data are reported as means ± SD or SE.

RESULTS

Exposure to Alkaloids and Effects on Health

Table 1 lists the EV and LB concentrations in the ryegrass hay used in this study measured on d 1, 8, and 22. Mean EV and LB in SE+ were 851 and 884 µg/kg DM, respectively, whereas these alkaloids were below the LD in the SE– hay. Over the course of the study, the mean hay consumption was 39 kg DM/ewe whatever the hay treatment, whereas the mean BW of ewes was 80 and 79 kg in SE– and SE+, respectively. The average hay moisture was 10.2%, which enabled calculation of mean intake of EV and LB of 14.9 and 15.5 µg/kg BW, respectively, in ewes fed the SE+ hay.

No animals died during the study, and no symptoms of toxicity were observed. No difference in hay consumption was observed between the 2 hays, and the ewes' BW remained constant throughout the study period in both SE– and SE+. Milk production decreased with time (P = 0.0002), whereas protein contents remained constant and fat contents increased (P = 0.01). However, no difference due to the hay distributed was observed in milk production or in the fat and protein contents in the milk (Table 1).

A postmortem examination at the end of the study did not reveal any pathological alteration, and no difference due to the hay distributed was observed in the weight of the carcass, liver, kidneys, and adrenals (data not shown).

Temperatures and Thermocirculation Index

Figure 1 shows core body temperature, skin temperature, air temperature, and the TCI in the ewes fed SE– and SE+. The core body temperature remained constant throughout the study whatever the hay used, even though air temperature varied from 15 to 5°C and skin temperature varied from 32 to 17°C (Fig. 1A). Because of the low stocking density and the size of the barn, the variations in the inside temperature were due to variations in outside temperature. The variations in skin temperature generally tracked variations in air temperature with no difference due to the hay fed. These changes led to variations in the TCI (Fig. 1B). But although TCI varied with time (P = 0.001), no difference due to the hay fed was observed.

Plasma Prolactin and Cortisol Concentrations and Biochemistry

Plasma PRL and cortisol concentrations are shown in Fig. 2. Because both parameters appeared to vary with time, a linear model was used to assess the effect of hay consumption on these variations. Concerning the concentration of PRL, the model revealed differences between SE+ and SE–, with an effect of time ($P < 0.00001$ and $P < 0.002$, respectively), which led us to use a complementary range test (Wilcoxon) for individual comparison of means. In the SE– fed ewes, the PRL concentration was less on Day 28 than on Day 0 (P = 0.04), whereas in the SE+ fed ewes, the concentration of PRL was less on Days 7, 14, 21, and 28 ($P < 0.01$). Moreover, a significant difference between SE+ and SE– was observed on Days 7, 14, 21, and 28 ($P < 0.03$). A linear model was also used to assess the effect of SE+ and SE– on cortisol concentrations in plasma. The model revealed differences between the hay fed (P = 0.001), but there was no effect of time. Individual comparison

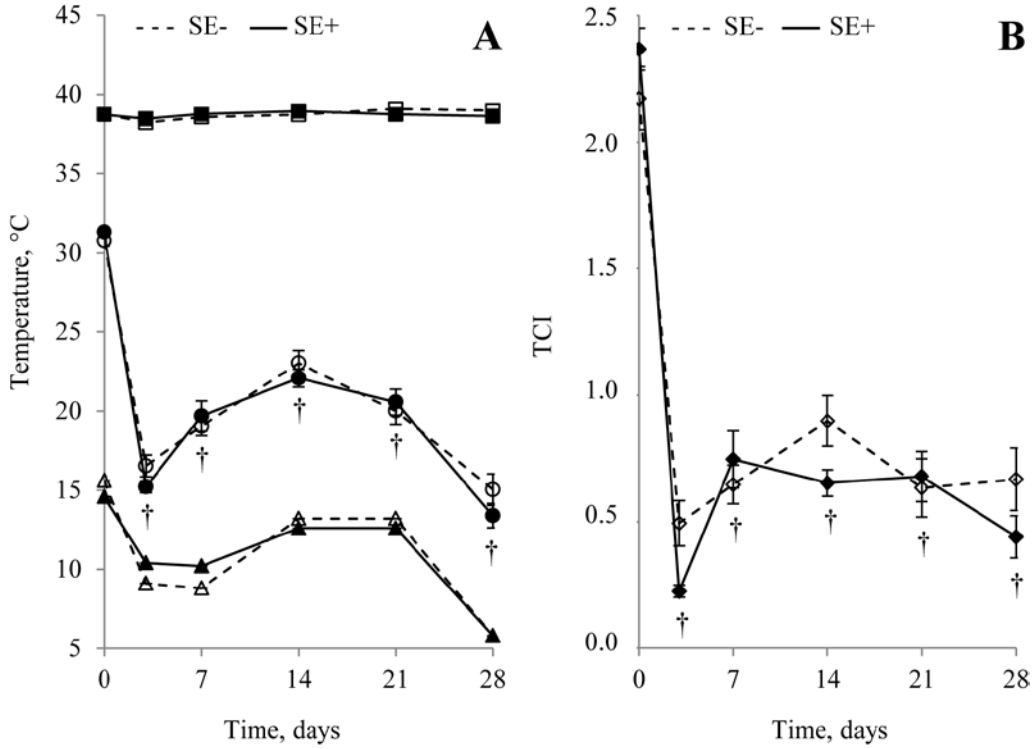

Figure 1. Effect of consuming endophyte-free ryegrass (SE–) or endophyte-infected ryegrass (SE+) on temperatures (A) and thermocirculation index (TCI; B) in ewes. Rectal (■), ear (●), and air temperatures (▲).†Significant difference from Day 0 (Wilcoxon, $P < 0.05$). Values are expressed as mean ± SE ($n = 8$).

of means (Wilcoxon test) revealed differences between SE+ and SE– only on Day 28 ($P = 0.0012$).

Table 2 lists hematocrit and biochemical parameters in ewes fed SE– or SE+ hay. A significant increase in the concentration of urea and a significant decrease in cholesterol and triglycerides were observed with time in both SE– and SE+ ($P < 0.000001$, $P < 0.05$, and $P <$ 0.00001, respectively). However, no difference between hays fed was observed, whatever the parameter analyzed.

Oxidative Damage and Antioxidant Enzyme Activities

Oxidative damage and antioxidant enzyme activities were measured in the plasma, liver, and kidneys

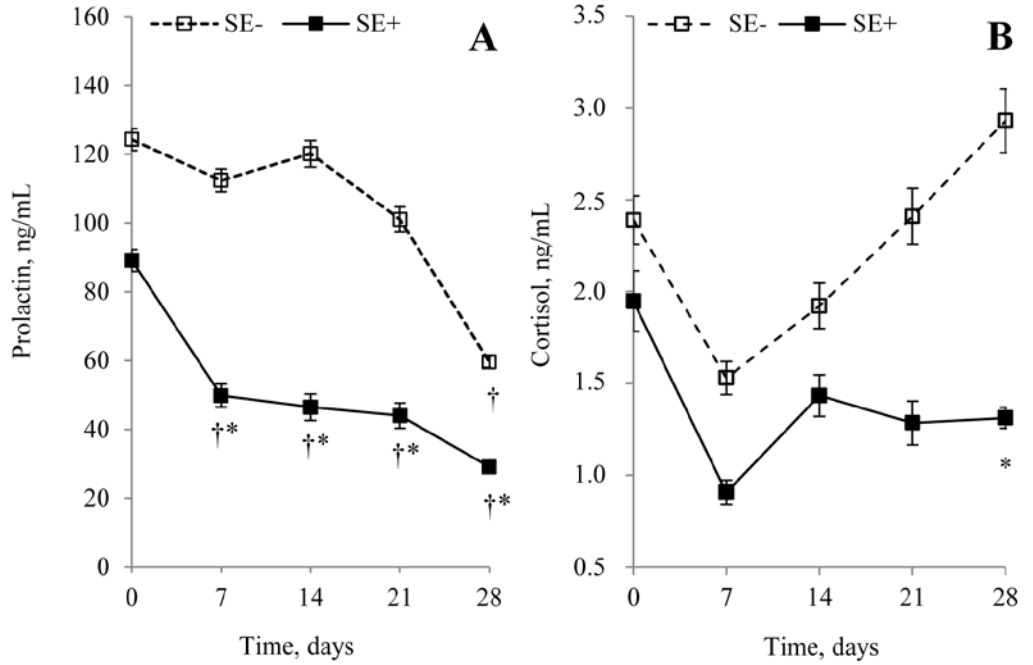

Figure 2. Plasma prolactin (A) and cortisol (B) concentrations in ewes fed endophyte-free ryegrass (SE–) or endophyte-infected ryegrass (SE+). †Significant difference from Day 0, *Significant difference between groups on the same day (Wilcoxon, $P < 0.05$). Values are expressed as mean ± SE (n = 8).

Table 2. Biochemical parameters in the plasma of ewes fed endophyte-free ryegrass (SE–) or endophyte-infected ryegrass (SE+). Values are expressed as mean ± SD (n = 8)

	SE–		SE +	
Parameter[1]	Day 0	Day 28	Day 0	Day 28
Hematocrit, %	33.5 ± 3.2	33.5 ± 4.4	34.5 ± 1.5	34.8 ± 1.8
Sodium, mEq/L	145 ± 3	146 ± 7	144 ± 3	146 ± 9
Potassium, mEq/L	4.61 ± 0.47	4.56 ± 0.62	4.70 ± 0.45	4.43 ± 0.63
Protein, g/L	74.3 ± 3.4	75.0 ± 7.2	73.7 ± 7.7	77.7 ± 4.5
Urea, mmol/L	7.23 ± 0.79	11.3 ± 1.5†	7.21 ± 1.42	9.75 ± 1.61†
Creatinine, µmol/L	68.9 ± 3.8	71.3 ± 10.4	64.2 ± 7.62	68.7 ± 8.8
Cholesterol, mmol/L	2.10 ± 0.37	1.78 ± 0.54†	2.20 ± 0.34	1.93 ± 0.32†
Triglycerides, mmol/L	0.25 ± 0.05	0.15 ± 0.02†	0.23 ± 0.03	0.18 ± 0.05†
Total bilirubin, µmol/L	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
AST, UI/L	155 ± 48	134 ± 23	162 ± 27	174 ± 50
ALT, UI/L	20.2 ± 8.3	21.2 ± 6.3	22.7 ± 3.6	25.6 ± 7.7
CPK, UI/L	150 ± 20	135 ± 39	177 ± 49	170 ± 43
LDH, UI/L	1,093 ± 284	1,006 ± 144	1,154 ± 211	1,140 ± 102

[1]AST = aspartate aminotransferase; ALT = alanine aminotransferase; CPK = creatine phosphokinase; LDH = lactate dehydrogenase.

†Significant difference from Day 0 (Wilcoxon, $P < 0.05$).

(Table 3). Total glutathione and GSSG and malondialdehyde were not affected by exposure to SE+ whatever the tissue analyzed. Concerning enzyme activities, an increase in SOD activity was observed in the plasma (P = 0.006) and kidney (P = 0.04) but not in the liver of SE+ fed ewes. Catalase activity in the kidney also increased in the SE+ fed eyes (P = 0.01), whereas it remained unchanged in the plasma and liver. Glutathione reductase and glutathione peroxidase activities were not affected by the diet of SE+ hay, whatever the tissue analyzed.

Drug Metabolizing Enzyme Activities

The effects of 28 d of feeding SE+ hay on hepatic and renal drug metabolizing enzymes activities in ewes are listed in Table 4. An increase (P = 0.002) in methoxyresorufin *O*-dealkylase activity was observed in the liver of ewes fed with SE+ hay (+37%) whereas the activity decreased (P = 0.009) in the kidney (–40%). No other drug metabolizing enzyme activity changed in the liver as the result of consuming SE+ hay, and no activity of *N*-demethylation was detected in kidneys whatever the substrate used. In the kidneys, glutathione *S*-transferase activity measured with 1,2-dichloro-4-nitrobenzene as substrate decreased (P = 0.002) in the SE+ fed ewes whereas it was not affected when 1-chloro-2,4-dinitrobenzene was used. By contrast, when para-nitrophenol was used as substrate, a 2-fold increase (P = 0.001) in uridine diphosphate glucuronosyltransferase activity was observed in the kidneys of SE+ fed ewes.

Table 3. Effects of 28 d of feeding ewes endophyte-free ryegrass (SE–) or endophyte-infected ryegrass (SE+) on oxidative stress parameters. Values are expressed as mean ± SD (n = 8)

	Day 28	
Parameter[1]	SE–	SE+
Plasma		
MDA, nmol/(min · mg of plasma proteins)	7.92 ± 1.03	8.81 ± 1.21
SOD, milliunits/(min · mg of plasma proteins)	6.00 ± 0.74	7.37 ± 0.97*
CAT, ng CH_2O/(min · mg of plasma proteins)	152 ± 96	130 ± 18
GR, µmol NADPH/(min · mg of plasma proteins)	0.45 ± 0.10	0.46 ± 0.09
GPx, µmol NADPH/(min · mg of plasma proteins)	0.79 ± 0.22	0.76 ± 0.27
TGSH, pmol/mg of plasma proteins	3.44 ± 0.88	3.42 ± 0.86
Liver		
MDA, nmol/mg of cytosolic proteins	62.6 ± 23.6	52.4 ± 7.6
MDA, mmol/mg of microsomal proteins	6.34 ± 1.42	6.80 ± 0.42
SOD, units/(min · mg of cytosolic proteins)	14.4 ± 3.4	12.3 ± 2.2
CAT, µg CH_2O/(min · mg of cytosolic proteins)	506 ± 30	472 ± 32
GR, µmol NADPH/min/(min · mg of cytosolic proteins)	19.5 ± 4.1	21.4 ± 2.6
GPx, µmol NADPH/min/(min · mg of cytosolic proteins)	101 ± 8	108 ± 9
TGSH, µmol/mg of cytosolic proteins	20.2 ± 11.2	17.5 ± 6.7
GSSG, µmol/mg of cytosolic proteins	1.29 ± 0.71	1.40 ± 1.14
Kidneys		
MDA, nmol/mg of cytosolic proteins	76.8 ± 32.4	77.4 ± 29.8
MDA, mmol/mg of microsomal proteins	13.4 ± 4.22	8.52 ± 1.78
SOD, units/(min · mg of cytosolic proteins)	11.74 ± 2.56	14.36 ± 2.30*
CAT, µg CH_2O/(min · mg of cytosolic proteins)	67.3 ± 7.4	85.3 ± 15.0*
GR, µmol NADPH/min/mg of cytosolic proteins	75.5 ± 13.1	84.6 ± 22.9
GPx, µmol NADPH/(min · mg of cytosolic proteins)	250 ± 36	270 ± 42
TGSH, µmol/mg of cytosolic proteins	0.66 ± 0.14	0.78 ± 0.12
GSSG, µmol/mg of cytosolic proteins	0.03 ± 0.02	0.04 ± 0.03

[1]MDA = malondialdehyde; SOD = superoxide dismutase; CAT = catalase; GR = glutathione reductase; GPx = glutathione peroxidase; TGSH = total glutathione; GSSG = oxidized glutathione; CH_2O = formaldehyde; NADPH = nicotinamide adenine dinucleotide phosphate.

*Significant difference between groups (Student, $P < 0.05$).

Residues in Milk and Tissues

Residues of EV and LB were measured in the milk, liver, kidneys, muscle, fat, and brain of the ewes fed SE– or SE+ hay (Table 5). Concentrations of EV and LB were always below the LD (0.15 µg/kg) in the SE– fed ewes whatever the sample analyzed. Also, EV was below the LD in muscle and brain of SE+ fed ewes, whereas 2 and 3 samples of fat and milk, respectively, out of the 8 samples analyzed contained higher concentrations than the LD. All the samples of kidneys and liver had EV at an higher concentration than the LD, but only 2 and 5 samples, respectively,

Table 4. Effects of 28 d of feeding ewes endophyte-free ryegrass (SE–) or endophyte-infected ryegrass (SE+) on hepatic and renal drug metabolizing enzymes. Values are expressed as mean ± SD (n = 8)

	Day 28	
Parameter	SE–	SE+
Liver, g	1,246 ± 349	1,015 ± 388
O-dealkylation[1]		
Ethoxyresorufin	4.28 ± 1.92	4.24 ± 1.54
Methoxyresorufin	1.82 ± 0.28	2.50 ± 0.44*
Pentoxyresorufin	1.54 ± 0.42	1.52 ± 0.18
N-demethylation[2]		
Aminopyrine	112 ± 27	97 ± 25
Benzphetamine	101 ± 28	111 ± 18
Dimethylnitrosamine	25.2 ± 4.1	23.9 ± 5.7
Erythromycin	21.4 ± 8.2	18.3 ± 6.7
Ethylmorphine	54.9 ± 11.0	48.4 ± 7.6
Glutathione S-transferase		
CDNB[3]	1.02 ± 0.22	0.86 ± 0.14
DCNB[4]	1.30 ± 0.28	1.16 ± 0.26
UDPGT[5]		
PNP[6]	1.22 ± 0.20	1.16 ± 0.20
Kidneys, g	199 ± 41	204 ± 25
O-dealkylation[1]		
Ethoxyresorufin	1.70 ± 0.24	1.34 ± 0.46
Methoxyresorufin	2.90 ± 0.86	1.74 ± 0.30*
Pentoxyresorufin	0.40 ± 0.28	0.50 ± 0.20
Glutathione *S*-transferase		
CDNB[3]	0.24 ± 0.02	0.26 ± 0.04
DCNB[4]	0.30 ± 0.12	0.12 ± 0.04*
UDPGT[5]		
PNP[6]	0.20 ± 0.08	0.41 ± 0.06*

[1]Expressed in picomoles resorufin formed/(minute·milligram of microsomal proteins).

[2]Expressed in nanograms formaldehyde formed/(minute·milligram of microsomal proteins.

[3]CDNB = 1-chloro-2,4-dinitrobenzene; expressed in millimoles of 1-chloro-2,4-dinitrobenzene conjugated/(minute·milligram of cytosolic proteins).

[4]DCNB = 1,2-dichloro-4-nitrobenzene; expressed in micromoles of 1,2-dichloro-4-nitrobenzene conjugated/(minute·milligram of cytosolic proteins).

[5]UDPGT = uridine diphosphate glucuronosyltransferase.

[6]PNP = para-nitrophenol; expressed in micromoles of paranitrophenol conjugated/(minute·milligram of microsomal proteins).

*Significant difference between groups (Student, $P < 0.05$).

were higher than the LQ (0.5 μg/kg). The mean EV level in quantifiable samples in the liver was 0.68 μg/kg. Lolitrem B was below the LD in the brain of SE+ fed ewes, whereas 2 samples of the milk out of the 8 analyzed contained LB at a nonquantifiable level. The number of samples with LB at a concentration higher than the LQ varied strongly depending on the matrix measured. Only 1 and 2 samples of kidney and liver, respectively, were higher than the LQ, whereas 4 and 8 samples of muscle and fat, respectively, contained a quantifiable level of LB. The highest concentration of LB was observed in fat; the mean was 2.39 μg/kg.

DISCUSSION

Animal Monitoring and Production

Consumption of SE+ causes tremorgenic symptoms in several animal species that can lead to the death in the more severe cases (Zbib et al., 2014a). Because LB is known to produce tremors and was found as the most abundant alkaloid of the indole-diterpene group in SE+, it is recognized as the main alkaloid responsible for ryegrass staggers in livestock (Zbib et al., 2014a). A toxic threshold of 2,000 μg LB/kg DM for ryegrass staggers was reported in sheep (Dimenna et al., 1992; Tor-Agbidye et al., 2001). In the present study, no tremor was observed after feeding ewes with SE+ hay containing 884 μg LB/kg DM for 28 d in the stable, which was not surprising considering the toxic threshold of LB.

Ergovaline is an ergot alkaloid also found in SE+ (Repussard et al., 2013). It has vasomotor effects considered responsible for the signs of lameness and reduced growth observed in fescue toxicosis (Oliver et al., 1993; Bacon, 1995; Zbib et al., 2014a). In FE+, a threshold of 500 to 800 μg EV/kg was reported in sheep (Tor-Agbidye et al., 2001). Although EV has been found in SE+, signs of toxicity linked to this toxin are rarely observed (Easton et al., 1996). Indeed, the EV concentration in SE+ often represents only 10 to 15% of LB concentration, and toxic levels of LB are generally reached before toxic levels of EV (Auldist and Thom, 2000; Hovermale and Craig, 2001; Johnstone et al., 2012).

The EV level in the SE+ hay used in this study was 851 μg/kg DM. This value is high compared with levels reported in New Zealand and the United States in SE+ (Auldist and Thom, 2000; Hovermale and Craig, 2001) but agrees with what was observed in Europe and Australia (Cagaš et al., 1999; Bony et al., 2001; Reed et al., 2011). The feeding of this hay in lactating ewes for 28 d in the stable had no consequence on feed consumption, skin temperature, and TCI and did not led to pathological alteration. This can, in part, be explained by the high protein concentrate used to meet the animal's requirements that could have decreased the SE+ toxicity (Bohnert and Merrill, 2006). However, this result was surprising considering the toxic threshold of EV in FE+ and the decreases of feed consumption, BW, skin temperature, and TCI observed in lactating ewes fed in the same experimental conditions with an FE+ hay containing 497 μg EV/kg DM and a similar level of protein concentrate in feed (Zbib et al., 2014b). It suggests that the toxic threshold of EV is not the same when the toxin is present in FE+ or SE+ hay. This observation agrees with previous studies conducted in lambs with feed containing FE+ and SE+ seeds (Gadberry et al., 2003). The diet

Table 5. Ergovaline and lolitrem B and concentrations in tissues and milk in ewes fed with endophyte-infected ryegrass after 28 d of exposure

	Ergovaline					Lolitrem B				
Matrix	n < LD[1]	LD < n < LQ	>LQ[2]	Mean ± SD[3]	Maximum[3]	n < LD[1]	LD < n < LQ	>LQ[4]	Mean ± SD[3]	Maximum[3]
Milk	4	4	0	–	–	6	2	0	–	–
Liver	0	3	5	0.68 ± 0.15	0.86	4	2	2	0.71 ± 0.15	0.81
Kidneys	0	6	2	0.53 ± 0.04	0.56	7	0	1	–	0.6
Muscle	8	0	0	–	–	0	4	4	0.77 ± 0.17	0.95
Fat	6	2	0	–	–	0	0	8	2.39 ± 1.67	4.78
Brain	8	0	0	–	–	8	0	0	–	–

[1]LD = limit of detection: 0.15 μg/L or 0.15 μg/kg for milk and tissues, respectively.

[2]LQ = limit of quantitation: 0.7 μg/L for milk, 0.8 μg/kg for fat and brain, and 0.5 μg/kg for liver, kidneys, and muscle.

[3]In micrograms/liter or micrograms/kilogram for milk and tissues, respectively.

[4]LQ = 0.5 μg/L or 0.5 μg/kg for milk and tissues, respectively.

done with FE+ seeds was more toxic than the diet done with SE+ seeds (results observed on feed intake, BW gain, skin temperature, and TCI) although the EV concentrations in the 2 diets were nearly the same (530 and 610 μg EV/kg, in FE+ and SE+ diet, respectively). This observation led Gadberry et al. (2003) to hypothesize that other alkaloids present in FE+ but not in SE+ increase toxicity. However, this hypothesis cannot be done in this study conducted with hay. Indeed, analysis of ergotamine, ergocryptine, ergocornine, ergosine, ergometrine, ergocristine, and their -inine forms revealed that these compounds were below 3 μg/kg DM in both FE+ and SE+ hays (Zbib et al., 2014b). This last result agrees with data on FE+ demonstrating that EV represented 80 to 100% of ergopeptides analyzed in forages (TePaske et al., 1993). Interestingly, studies conducted in sheep grazing different cultivars of SE+ has revealed that the toxicity of EV was masked in endophyte-infected perennial ryegrass when high level of LB are present. By contrast, signs of EV toxicity were observed in 1 Endosafe cultivar that was unable to produce LB. This cultivar was withdrawn from the market (Fletcher, 2013).

Plasma Prolactin Concentration

In this study, PRL concentrations decreased with time whatever the hay fed, which is common during lactation and during short photoperiods (Convey, 1974; Carr and Land, 1982). This decrease agrees with previous study conducted in ewes fed with endophyte-free tall fescue hay under the same conditions (Zbib et al., 2014b). However, the PRL decrease was more pronounced in ewes fed SE+ hay from Day 7 to 28 than in ewes fed SE–. A PRL decrease during EV exposure has been linked to the block of the dopaminergic receptors of the pituitary cells (Schillo et al., 1988; Siegel et al., 1989; Strickland et al., 1994). It is often reported in animals fed with FE+ but is less common with SE+ (Zbib et al., 2014a). In the present study, the PRL decrease was of 56 and 51% on Days 7 and 28, respectively. In lactating ewes fed FE+ hay containing 497 μg EV/kg, the PRL decrease was 66 and 73%, also on Days 7 and 28, respectively (Zbib et al., 2014b). This observation agrees with what was observed in lamb fed FE+ and SE+ seeds in the diet (Gadberry et al., 2003). The PRL decrease was of 83 and 59% in diets containing FE+ and SE+ seeds at final EV concentrations of 610 and 530 μg/kg, respectively (Gadberry et al., 2003). The more important decrease of PRL observed in ewes fed SE+ hay has no consequence on milk production, in agreement with data suggesting that small amounts of PRL remaining in the circulation may be sufficient to maintain functional integrity in the gland (Forsyth, 1986).

Cortisol, Biochemistry, and Oxidative Stress Parameters

A significant difference in the concentration of cortisol was observed between SE– and SE+ fed ewes on Day 28, but this result is difficult to explain. No effect of time was observed whatever the hay consumption was, and the concentrations measured were within the expected range in ewes (Negrão and Marnet, 2003). Moreover, previous studies failed to reveal an effect of SE+ feeding on this parameter (Watson, 2000; Looper et al., 2006).

No effect of SE+ hay was observed on biochemistry in this study, which is in agreement with results of previous studies even when staggers occurred (Miyazaki et al., 2007). Activities of enzymes that are markers of cell damage are increased only when severe staggers occurred (Benkhelil et al., 2004).

No data is available concerning the effect of SE+ on oxidative damage, whereas a decrease in antioxidant defense mechanisms is observed in FE+ fed animals, especially under heat stress (Lakritz et al., 2002; Spiers et al., 2005; Bhusari et al., 2006; Burke et al., 2007; Settivari et al., 2008). The results of the present study suggest a slight increase in the activity of some

enzymes involved in defense mechanisms against oxidative damage in ewes fed SE+ hay. By contrast, a moderate decrease in the mechanism of defense against oxidative damage was observed in lactating ewes fed FE+ hay containing 497 μg EV/kg (Zbib et al., 2014b).

Drug Metabolizing Enzymes

The effects of feeding SE+ hay on drug metabolizing enzyme activities were slight and differed depending on the tissue analyzed and the activity measured. Among them, the decrease of glutathione *S*-transferase activity alone agrees with what was observed on drug metabolizing enzymes activities in ewes fed with FE + hay (Zbib et al., 2014b). Especially, the increase in erythromycin *N*-demethylase activity that was observed in lactating ewes fed with FE+ was not observed in this study (Zbib et al., 2014b). No data is available concerning the effect of SE+ on drug metabolizing enzymes, whereas several studies were conducted with FE+ seeds in rodents (Zbib et al., 2014a). The differences observed in lactating ewes fed with SE+ or FE+ hays could be due to the presence of alkaloids other than EV in SE+. Indeed, although the effect of LB on drug metabolizing enzyme activities is unknown, studies conducted with indole alkaloids suggested that these compounds interact with drug metabolizing enzymes. Among them, rutaecarpine, an indole alkaloid of *Evodia rutaecarpa*, has been shown to increase methoxyresorufin *O*-dealkylase activity and increase uridine diphosphate glucuronosyltransferase expression in mice (Jeon et al., 2006; Zhu et al., 2013). Both activities that were increased in SE+ fed ewes in this study.

Residues in Milk and Tissues

The level of EV in the ewes' milk measured in this study was below the LQ, in agreement with kinetics of EV in lactating goats that demonstrated an excretion rate in milk of only 0.05% (Durix et al., 1999). The liver was the tissue with the highest EV level, probably because of its anatomical position after absorption of the toxin, whereas the kidneys contained fewer EV, in agreement with data demonstrating high metabolism of ergot alkaloids leading to almost complete absence of the parent drug in urine (Maurer et al., 1983; De Lorme et al., 2007). The LB concentration in milk was also below the LQ. This result agrees with what was observed in dairy cows, with only 0.23% of the amount of LB ingested being excreted in milk (Finch et al., 2013). Fat was the tissue that contained the highest level of LB, with all the samples above the LQ. The mean concentration was 2.39 μg/kg. The relative high level of LB in fat compared with the other tissues was already reported in cattle, both in dairy cows and steers (Miyazaki et al., 2004, 2007; Finch and Babu, 2012; Shimada et al., 2013). Taken together, these results suggested that EV and LB persistence in tissues and excretion in milk were very low. The use of a HPLC–mass spectrometry method (Smith et al., 2009) for an unequivocal identification of the mycotoxins and the possible biotransformation products of EV and LB is necessary to confirm these results.

In conclusion, feeding SE+ hay containing 851 and 884 μg/kg of EV and LB, respectively, to lactating ewes for a period of 28 d in the stable had no consequence for animal health or milk production. Analysis of milk and tissues revealed the presence of EV and LB at low levels in SE+ fed ewes, with most of the samples analyzed being below the LQ. Concerning biological effects in ewes, a small decrease in PRL was observed from Day 7 to 28, confirming exposure to EV. The measurement of drug metabolizing enzyme activities suggests complex effects of the toxins, different from those previously observed by feeding FE+ hay. Taken together, these results strongly suggest that the toxic threshold of EV differs in FE+ and SE+. Although further studies are necessary to understand the effect of alkaloids on drug metabolizing enzymes activities, these alterations may contribute to the difference in toxicity observed.

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