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Enterococci inoculated silages: Effect on rumen fermentation and lipid metabolism *in vitro*

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The effect of providing diets containing grass silage (12.8 g of DM/day) and barley grain (3.2 g of DM/day) or maize silage (12.8 g of DM/day) and lucerne hay (3.2 g of DM/day) into RUSITEC (rumen simulation technique) in which the grass or maize were uninoculated (GS, MS) or inoculated with strains *Enterococcus faecium* EF2/3s (GS + EF2/3s, MS + EF2/3s) or *E. faecium* EF26/42 (GS + EF26/42, MS + EF26/42) on rumen fermentation patterns and lipid metabolism was examined. The inoculated GS diets decreased proportion of acetate (P < 0.001) and increased the proportion of *n*-butyrate (P < 0.001) compared to control. The inoculated MS diets increased proportion of acetate, *n*-butyrate and decreased proportion of propionate, methane and ammonia nitrogen. The efficiencies of microbial protein synthesis were increased by the inoculated GS and MS diets. The concentration of *cis*9 *trans*11 C18:2 (CLA) and *trans*11 C18:1 (TVA) in effluent was similar during fermentation in both GS and MS diets, with the tendency of higher values of CLA and TVA in MS + EF2/3s diet. The biohydrogenation (BH) of C18:1 and C18:2 was decreased (P < 0.05) in MS + EF2/3s diet, but BH of C18:1 and C18:3 was increased (P < 0.05) in MS + EF26/42 diet, while BH of these FA in GS diets were unchanged.

Key words: Fatty acids, grass silage, maize silage, rumen fermentation, rumen simulation technique.

INTRODUCTION

Forages are an important dietary source of α-linolenic acid (C18:3*n*-3, ALA) and linoleic acid (C18:2*n*-6, LA) and silage conserve the forages with minimal loss of nutritive value by fermentation of soluble carbohydrates in an anaerobic environment into organic acids, preferably lactic acid, which reduce pH (Saarisalo et al., 2007). The effect of ensiling on total fatty acid (FA) contents of forages is inconsistent (Elgersma et al., 2003; Boufaied et al., 2003). It is widely accepted that certain microbiota such as lactobacilli, lactococci, propionibacteria,

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Abbreviation: ALA, α-linolenic acid, C18:3*n*-3; **ADF,** acid detergent fibre; **BG,** barley grain; **BH,** biohydrogenation; **CP,** crude protein; **CLA,** conjugated linoleic acids, *cis*9 *trans*11 C18:2; **dADF,** degraded acid detergent fibre; **DM,** dry matter; **dNDF,** degraded neutral detergent fibre; **E,** energetic efficiency; **EF2/3s,** *Enterococcus faecium* EF2/3s; **EF26/42,** *E. faecium* EF26/42; **EMS,** efficiency of microbial protein synthesis; **FA,** fatty acids; **GS,** grass silage; **IVDMD,** *in vitro* dry matter digestibility; **LA,** linoleic acid; **LAB,** lactic acid bacteria; **LCFA,** long chain FA (> C18:0); **LH,** lucerne hay; **MCFA,** medium chain FA (C14:0 - C17:1); **MS,** maize silage; **NDF,** neutral detergent fibre; **N_M,** N incorporated by microflora; **OMF,** organic matter fermented; **PUFA,** polyunsaturated fatty acids; **SCFA,** short chain fatty acids; **TVA,** *trans* vaccenic acid, *trans*11 C18:1.

bifidobacteria and enterococci are able to form conjugated linoleic acid (CLA, *cis*9, *trans*11 C18:2) from LA (Sieber et al., 2004; Marciňáková, 2006). Lactic acid bacteria commonly found in forages have the ability to biohydrogenate LA and ALA, to isomerize LA into CLA and to reduce CLA into *trans*10 C18:1 FA (Ogawa et al., 2005; Kishino et al., 2009). It is also known that strains belonging to the genera *Enteroccocus*, *Pediococcus*, *Propiniobacterium* and *Lactobacillus* produce considerable amounts of CLA (Kishino et al., 2002). In addition, the screening of microorganisms at our Institute (72 species of lactobacilli and enterococci) showed that some lactobacilli (4 species) and enterococci (13 species) isolated from rumen fluid and silages were able to convert LA to CLA in special growth medium *in vitro* (Marciňáková, 2006). Previously, three microorganisms (*E. faecium* CCM4231, *L. plantarum* CCM4000 and *L. fermentum* LF2) of this screening were used as inoculants during ensiling of grass and maize (Jalč et al., 2009a, 2009b). In addition, the effect of inoculated grass and maize silage diets on rumen fermentation and lipid metabolism in artificial rumen (RUSITEC) was studied (Jalč et al., 2009c, 2009d).

The aim of the present study was to increase the concentration of CLA and TVA (*trans*-vaccenic acid, *trans*11 C18:1) and decrease biohydrogenation of grass and maize silage diets during rumen fermentation either unionculated (used as a negative control, not a treatment) or inoculated with two silage inoculants, *Enterococcus faecium* EF2/3s or *Enterococcus faecium* EF26/42 using artificial rumen RUSITEC.

MATERIALS AND METHODS

Treatments, material and ensiling

Three treatments were performed for grass silage (GS) and maize silage (MS): untreated silage (GS or MS, control), without inoculant treated with distilled water; grass or maize treated (inoculated) by the strain *E. faecium* EF2/3s (GS + EF2/3s or MS + EF2/3s); and grass or maize inoculated with the strain *E. faecium* EF26/42 (GS + EF26/42 or MS + EF26/42), respectively. Both *E. faecium* inoculants belong to the lactic acid bacteria (LAB); they produce bacteriocins and produce considerable amounts of CLA (Marciňáková, 2006). The inoculants were isolated at our Institute (*E. faecium* EF2/3s is grass silage isolate; *E. faecium* 26/42 is calf rumen content isolate). For ensiling, a fresh culture of each inoculant bacterial strain was diluted in Ringer solution to a population of at least 10^9 cfu/ml. The diluted inoculants were sprayed on at 10 ml per kg of grass or maize. The control was sprayed on 10 ml of distilled water per kg of grass or maize. For ensiling, the first cut of orchardgrass (*Dactylis glomerata)* and maize (*Zea mays* L.) was wilted for 16 h, then chopped to a length of 20 mm with a forage chopper and sprayed with the bacterial inoculants. Prior to start the RUSITEC experiments, representative samples of the raw material (untreated chopped grass and maize) were taken for microbiological and chemical analyses before the filling of the jars as well as at the end of ensiling and results were published in Váradyová et al. (2013).

Fermentation system of the artificial rumen

We used RUSITEC for fermenting the experimental feed rations. The fermentation equipment included four fermentation vessels, each having a volume of 850 ml (Jalč et al., 2009c, 2009d). The vessel inoculum was obtained from four ruminally cannulated Slovak merino rams (age 7 years, mean body weight 45.0 ± 2.5 kg) which had been fed 1040 g dry matter (DM) of meadow hay and 260 g DM of crushed barley grains in two equal meals per day. The rams were housed separately in pens, with free access to water and a mineral mixture for sheep. The fermentation inocula (that is, solid and liquid) were collected through the rumen cannula before a morning feeding and then pooled. On the first day of the experiment, 450 ml of strained rumen fluid and 400 ml of artificial saliva were put into each fermentation vessel (McDougall, 1948). Squeezed particulate rumen contents (100 g) were weighed into a nylon bag (pore size 100 μm), which was then placed inside the feed container in each vessel together with a bag of feed. After 24 h, the bag with the original solid inoculum was withdrawn and a bag with feed was added. On subsequent days, the bag that had remained for two days in each vessel was replaced by a new bag with feed. Therefore, two bags were present at any given time, and one of them was removed each day to give a 48 h incubation. Continual infusion of artificial saliva supplemented with microelements (mg/L; $ZnSO_4$: 1.92; $MnSO_4$: 1.02; $CoSO_4$: 0.06 and pH 8.4) was maintained at the rate of 625 ± 11.0 ml/day through each vessel during the experiments.

Experiment design

The four experiments were conducted as repeated measures in which the four treated feed rations and their respective control were used (GS versus GS + EF2/3s, or GS versus GS + EF26/42, and MS versus MS + EF2/3s, or MS versus MS + EF26/42, respecttively). In the first and second experiment, four vessels received the grass silage diets, where two vessels of each diets were supplied with 12.8 g of DM of grass silage (GS + EF2/3s or GS + EF26/42) and 3.2 g of DM of crushed barley grain daily and the other two vessels received uninoculated grass silage (GS, control) and 3.2 g of DM of crushed barley grain. In the third and fourth experiment, four vessels received the maize silage diets, where two vessels of each diets were supplied with 12.8 g of DM of MS silage (MS + EF2/3s or MS + EF26/42) and 3.5 g of DM of lucerne hay and the other two vessels received uninoculated maize silage (MS, control) and 3.5 g of DM of lucerne hay. The incubation experiments in RUSITEC lasted 12 days and consisted of a 6-day adaptation period followed by a 6 day of treatment period, with sampling and measures on the last 6 days. The numbers of samples were duplicate from 6 experimental days, thus average values from 12 samples are in Tables 2 to 5. Immediately after sampling, the pH of the vessel contents was measured. The experiments were repeated with the same design.

Measurements and chemical analysis

The nutrient composition, fermentation characteristics, fatty acids and microbial counts of uninoculated and inoculated silages after ensiling are in Table 1. These silages were used as the components of a ration together with lucerne hay (MS) or barley grain (GS) for the RUSITEC experiments. Analyses of FA before the RUSITEC fermentations were done with 0.5 g of each substrate, and the total input was calculated per actual amounts of diet substrates used in the fermentation vessels. After fermentation in the RUSITEC systems, the bags of feed (12 bags/feed treatment)

Table 1. Chemical composition of dietary ingredients.

Item	Grass silage			BG	Maize silage			LH
	GS	EF2/3s	EF26/42		MS	EF2/3s	EF26/42	
DM (g/kg)	285	272	279	878	320	314	318	913
N (g/kg DM)	16.0	17.5	17.5	19.7	11.7	11.7	11.4	38.9
CP	100	110	110	123	73.2	73.1	71.2	243
NDF	670	677	682	162	410	417	388	496
ADF	431	410	376	82.2	245	245	218	349
Fat	31.6	29.3	28.4	24.0	30.0	24.7	29.2	17.0
Ash	55.9	59.4	56.6	25.9	52.1	59.1	45.4	95.8
IVDMD	542	522	438	890	617	687	684	Nd
pH	4.47	4.29	4.21	Nd	3.79	3.78	3.77	Nd
Lactate	72.1	70.7	57.0	Nd	26.2	33.8	71.0	Nd
Acetate	25.6	16.7	13.6	Nd	19.4	20.9	18.9	Nd
Propionate	16.3	11.4	10.4	Nd	0.00	0.00	0.00	Nd
NH_3N (g/kg N)	83.1	56.7	86.7	Nd	43.0	48.2	52.5	Nd
C14:0 (g/kg FA)	14.8	14.0	13.2	3.93	8.20	9.51	6.97	12.7
C16:0	177	231	221	204	238	236	225	256
C18:0	21.5	42.7	23.3	28.5	29.5	30.7	32.3	55.8
C18:1*n*-9	33.8	43.7	48.5	154	193	180	196	58.8
C18:2*n*-6	112	120	181	522	366	348	374	178
C18:3*n*-3	314	345	320	38.1	122	144	120	269
MCFA	214	269	253	212	251	259	244	274
LCFA	763	706	730	785	728	724	745	625
Inoculants log10 (cfu/g)	Nd	4.62	3.76	Nd	Nd	<1.0	1.60	Nd

GS, grass silage; MS, maize silage; EF2/3s, *Enterococcus faecium* EF2/3s; EF26/42, *E. faecium* EF26/42; BG, barley grain; LH, lucerne hay; DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; IVDMD, *in vitro* dry matter digestibility; FA, fatty acids; MCFA, medium chain FA (C14:0 - C17:1); LCFA, long chain FA (> C18:0); Nd, not determined.

were also chemically analyzed in triplicates immediately after collection. Undigested feed samples in bags were treated by oven drying at 60°C, 48 h for assay of DM. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analyzed using the FiberTec 2010 (Teactor Comp., Höganäs, Sweden) (Van Soest et al., 1991). ADF was expressed inclusive of residual ash. NDF was assayed with sodium sulfite, without heat-stable amylase, and expressed inclusive of residual ash. Standard methods were used to determine the ash (AOAC Official Method 942.05), nitrogen (AOAC Official Method 968.06), fat (AOAC Official Method 983.23) and crude protein (AOAC Official Method 990.03) (AOAC, 1990). During RUSITEC fermentation, the gas produced was collected into special bags (Tesseraux GmbH, Bürstadt, Germany), and the methane concentrations were analyzed in a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc., Shelton, CN, USA). Liquid effluent was collected into flasks placed in an ice bath, and samples were taken for short chain fatty acids (SCFA, C2-C4), ammonia N and FA analyses. Daily production of SCFA (total and individual) was analyzed using gas chromatography (Cottyn and Boucque, 1968). Ammonia N concentrations were measured by the microdiffussion method (Conway et al., 1962).

Fatty acid analysis

Volumes of 100 ml of liquid effluents were lyophilized and weighted. These amounts of weighted samples were calculated on total amount of produced effluents/day. The fatty acids in fresh maize and maize silages were determined in lyophilised samples. Samples were freeze-dried using a ThermoSavant Micromodulyo freeze-drier (Thermo Savant MicroModulyo, NY, USA), placed in pre-cleaned high density polyethylene flasks, and kept in the dark at laboratory temperature until analyzed. Lipids from freeze-dried maize and maize silages were extracted from 0.5 g of sample with a 2:1 mixture of chloroform:methanol, with purification of samples using 20% HCl (Bligh and Dyer, 1959). The extracted lipids were dissolved in 1 ml of hexane and 1 ml of internal standard (that is, tridecanoic acid; Fluka, Chemie GmbH, Buchs, Switzerland). Subsequently, 2 ml of transesterification reagent (that is, 1N methanolic sodium methoxide; Fluka, Chemie GmbH, Buchs, Switzerland) was added to this mixture (Baše, 1978). The mixture was kept in a water bath at 50°C for 30 min. After the addition of 3 ml of 3 N methanolic HCl (Supelco, Bellefonte, PA, USA), the mixture was incubated in a water bath at 50°C for 1 h. To separate the hexane layer in the mixture, 1 ml of hexane and 1 ml of distilled water were added. Finally, the mixture was centrifuged at 200 × *g* for 5 min at laboratory temperature. The upper hexane layer was used for determination of fatty acid methyl esters by gas chromatography. Samples were injected by a splitless injector into a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc. Shelton, CN, USA) equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, Inc., Santa

Table 2. Effect of grass silage diet (GS) on the rumen fermentation patterns in RUSITEC effluent.

Item	Grass silage			SEM	Significance		
	GS	EF2/3s	EF26/42		D	T	D × T
Degradability DM (g/kg)	574	573	571	8.78	Ns	**	Ns
dNDF (g/kg DM)	443	429	378	10.8	*	Ns	Ns
dADF (g/kg DM)	340	317	298	22.5	Ns	**	Ns
pH	6.43	6.58	6.55	0.08	*	Ns	Ns
SCFA (mmol/day)	51.3	51.9	49.0	1.83	Ns	**	Ns
Mol SCFA/kg digested DM	6.05	5.50	5.43	0.101	Ns	Ns	Ns
Acetate (mmol/day)	33.0	29.8	27.8[a]	1.08	**	**	Ns
Propionate (mmol/day)	10.1	9.28	9.59	0.27	Ns	Ns	Ns
n-Butyrate (mmol/day)	6.01	8.56[b]	7.39[b]	0.30	***	Ns	Ns
A/P	3.26	3.01	2.83	0.140	**	***	Ns
Acetate (molar %)	62.0	56.3[b]	56.7[b]	0.68	***	**	Ns
Propionate (molar %)	19.2	19.1	19.9	0.51	Ns	**	Ns
n-Butyrate (molar %)	11.7	17.9[c]	16.2[c]	0.37	***	Ns	Ns
E (molar %)	74.4	75.2[a]	75.6[a]	0.26	**	Ns	Ns
Total gas (ml/day)	4330	4164	4140	191.7	Ns	Ns	Ns
Methane (mmol/day)	9.62	7.30	7.27	0.801	Ns	Ns	Ns
Ammonia N (mg/l)	218.3	150.2[b]	151.5[b]	22.1	**	Ns	Ns
EMS = N_M/ OMF (mg/g)	22.1	27.2[a]	29.3[a]	0.75	**	Ns	Ns

EF2/3s, *Enterococcus faecium* EF2/3s; EF26/42, *E. faecium* EF26/42; DM, dry matter; dNDF, degraded neutral detergent fibre; dADF, degraded acid detergent fibre; SCFA, short chain fatty acids; A/P ratio, acetate to propionate ratio; E, energetic efficiency of SCFA's; EMS, efficiency of microbial protein synthesis; N_M, N incorporated by microflora; OMF, organic matter fermented; D, diets; T, time; SEM, standard error of the means; *P < 0.05, **P < 0.01, ***P < 0.001; [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 express differences between control (GS) and experimental diets.

Table 3. Daily output of fatty acids (g/100g per vessel) and biohydrogenation of FA (%) in grass silage (GS) diet in RUSITEC effluent.

Item	Grass silage			SEM	Significance		
	GS	EF2/3s	EF26/42		D	T	D × T
C14:0 myristic	22.6	24.9	13.7	3.36	***	**	***
C16:0 palmitic	186	186	97.8	13.6	***	Ns	**
C18:0 stearic	293	233	186	19.2	***	Ns	*
C18:1*n*-9 oleic	11.0	7.22	6.68	0.73	***	Ns	*
C18:1 *t*11-TVA	35.2	28.4	30.8	2.33	***	Ns	***
C18:2*n*-6 linoleic	10.7	7.45[a]	6.78[a]	0.63	***	**	Ns
C18:2, *c*-9 *t*-11-CLA	0.39	0.35	0.26	0.06	*	**	**
C18:3*n*-3 α-linolenic	5.84	5.18	4.49	0.86	*	**	**
Total FA	767	656[a]	494[c]	15.9	***	**	Ns
Biohydrogenation of FA							
C18:1*n*-9 oleic	68.7	79.8	88.3	4.95	Ns	Ns	Ns
C18:2*n*-6 linoleic	93.3	93.4	96.2	1.15	Ns	Ns	Ns
C18:3*n*-3 α-linolenic	98.5	98.3	98.9	0.28	Ns	Ns	Ns
Total C18	86.8	90.8	94.5	1.91	Ns	Ns	Ns

EF2/3s, *Enterococcus faecium* EF2/3s; EF26/42, *E. faecium* EF26/42; TVA, *trans* vaccenic acid, *trans*11 C18:1; CLA, conjugated linoleic acids, *cis*9 *trans*11 C18:2; FA, fatty acids; D, diets; T, time; SEM, standard error of the means. Total FA's also included C8:0-C13:0, C16:1, C17:0, C20:0-C20:5, C21:0, C24:0; *P < 0.05, **P < 0.01, ***P < 0.001; [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 express differences between control (GS) and experimental diets.

Table 4. Effect of maize silage (MS) diets on the rumen fermentation patterns in RUSITEC effluent.

Item	Maize silage			SEM	Significance		
	MS	EF2/3s	EF26/42		D	T	D × T
Degradability DM (g/kg)	540	515	508	23.0	Ns	Ns	Ns
Degradability OM (g/kg)	476	521	410	30.2	Ns	Ns	Ns
dNDF (g/kg DM)	401	428	431	21.3	Ns	Ns	Ns
dADF (g/kg DM)	242	233	251	16.1	Ns	Ns	**
pH	6.56	6.64	6.55	0.063	Ns	Ns	Ns
SCFA (mmol/day)	53.6	52.0	52.4	1.95	Ns	Ns	Ns
mol SCFA/kg digested DM	6.78	6.46	6.46	0.25	Ns	Ns	Ns
Acetate (mmol/day)	31.8	32.4	33.3	1.17	Ns	Ns	Ns
Propionate (mmol/day)	8.98	7.84	7.65	0.92	Ns	Ns	Ns
n-Butyrate (mmol/day)	5.92	6.63	6.55	0.36	Ns	Ns	Ns
A/P	3.40	4.19[b]	4.37[c]	0.13	***	**	Ns
Acetate (molar %)	61.8	62.3	63.3[b]	1.12	**	***	***
Propionate (molar %)	18.2	15.0[b]	14.5[b]	1.43	***	**	Ns
n-Butyrate (molar %)	11.7	12.8[a]	12.7[a]	0.84	**	***	***
Total gas (ml/day)	3703	3780	4051	88.9	Ns	Ns	Ns
Methane (mmol/day)	6.32	3.90[a]	3.81[b]	0.84	***	Ns	Ns
Ammonia N (mg/l)	232.4	154.7[b]	174.7[a]	21.1	***	Ns	Ns
N_M (mg/day)	81.1	105[a]	106[b]	8.87	***	***	**
EMS = N_M/ OMF (mg/g)	16.2	20.2[b]	20.7[b]	2.03	**	***	***

EF2/3s, *Enterococcus faecium* EF2/3s; EF26/42, *E. faecium* EF26/42; DM, dry matter; OM, organic matter; dNDF, degraded neutral detergent fibre; dADF, degraded acid detergent fibre; SCFA, short chain fatty acids; A/P ratio, acetate to propionate ratio; N_M/ OMF, efficiency of microbial protein synthesis; N_M, N incorporated by microflora; OMF, organic matter fermented; D, diets; T, time; SEM, standard error of the means; *P < 0.05, **P < 0.01, ***P < 0.001; [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 express differences between control (MS) and experimental diets.

Table 5. Daily output of fatty acids (g/100g per vessel) and biohydrogenation of FA (%) in maize diet (MS) in RUSITEC effluent.

Item	Maize silage			SEM	Significance		
	MS	EF2/3s	EF26/42		D	T	D × T
C14:0 myristic	9.96	9.89	10.8	0.523	Ns	**	***
C16:0 palmitic	167	167	174	6.5	Ns	Ns	Ns
C18:0 stearic	348	372	399	9.1	Ns	Ns	Ns
C18:1*n*-9 oleic	39.5	28.0[b]	29.8[b]	5.71	***	***	***
C18:1 *t*11-TVA	12.5	12.9	10.6	0.78	Ns	Ns	Ns
C18:2*n*-6 linoleic	26.9	22.3	28.5	2.17	Ns	Ns	Ns
C18:2, *c*-9 *t*-11-CLA	0.064	0.179	0.079	0.31	Ns	Ns	Ns
C18:3*n*-3 α-linolenic	4.09	4.18	4.43	0.097	Ns	Ns	Ns
Total FA	732	734	792[b]	4.10	***	Ns	Ns
Biohydrogenation of FA							
C18:1*n*-9 oleic	71.2	62.5[a]	78.3[a]	0.85	**	Ns	Ns
C18:2*n*-6 linoleic	87.2	84.1[a]	89.6	0.83	**	**	*
C18:3*n*-3 α-linolenic	93.5	93.8	95.4[a]	0.21	*	Ns	Ns
Total C18	84.0	81.5	87.3[a]	0.59	*	Ns	Ns

EF2/3s, *Enterococcus faecium* EF2/3s; EF26/42, *E. faecium* EF26/42; BH, biohydrogenation of fatty acids; TVA, *trans* vaccenic acid, *trans*11 C18:1; CLA, conjugated linoleic acids, *cis*9 *trans*11 C18:2; total FA's also included C8:0-C13:0, C16:1, C17:0, C20:0-C20:5, C21:0, C24:0; D, diets; T, time; SEM, standard error of the means; *P < 0.05, **P < 0.01, ***P < 0.001; [a]P < 0.05, [b]P < 0.01, [c]P < 0.001 express differences between control (MS) and experimental diets.

Clara, CA, USA) and a flame ionization detector (constant flow, hydrogen 40 ml/min, air 400 ml, 260°C).

Analyses of FA (0.5 µl methyl esters in hexane injected at a 30:1 split ratio) were carried out under a temperature gradient (130°C for 1 min; 130 to 170°C at program rate 6.5°C/min; 170 to 206°C at program rate 1°C/min; 206 to 240°C at program rate 34°C/min) with hydrogen as the carrier gas (flow 1.8 ml/min, velocity 44 cm/s, pressure 23.2 psi). The fatty acid methyl ester peaks were identified with a commercial mixture (Supelco 37 component FAME MIX, Supelco, Bellefonte, PA, USA) and quantified by the internal standard of tridecanoic acid (C13:0; Supelco, Bellefonte, PA, USA).

Calculations and statistical analyses

Energetic efficiency (E) of SCFAs, organic matter fermented (OMF), N incorporated by microflora (N_M) and efficiency of microbial protein synthesis (EMS = N_M/OMF in mg/g) were calculated from the stoichiometry of rumen fermentation (Ørskov et al., 1968; Demeyer and Van Nevel, 1979; Alves de Oliveira et al., 1997). Biohydrogenation (BH) of fatty acids (C18:1, C18:2 and C18:3) was calculated from the following equation as the difference between daily intake (daily input) and effluent flow (daily output) as a proportion of daily intake (Fievez et al., 2007):

$$\text{BH (\%)} = 100 \times \frac{\text{PUFA input (C18:1, C18:2 or C18:3)} - \text{PUFA output (C18:1, C18:2 or C18:3)}}{\text{PUFA input (C18:1, C18:2 or C18:3)}}$$

Statistical analysis was performed using analysis of variance (GraphPad Instat, GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of RUSITEC measurements (Tables 2 to 5) used analysis of variance as a repeated measures mixed model that represented the dietary groups (GS versus GS + EF2/3s, or GS versus GS + EF26/42 and MS versus MS + EF2/3s, or MS versus MS + EF26/42) and the six-time points of measurements. Effects included in the model were time (T), diet (D), interaction between time and diet (D × T). Differences between uninoculated GS or MS (control) and inoculated GS or MS were analyzed by two-way ANOVA with Bonferroni post-test. Differences were considered to be significant when P < 0.05.

RESULTS

Rumen fermentation parameters of grass silages in RUSITEC

The diet (D) affected the results of NDF, pH, acetate, *n*-butyrate, A/P ratio, energetic efficiency (E), ammonia N and the efficiency of microbial protein synthesis (EMS) in fermentation in the RUSITEC (Table 2). The molar proportions of acetate and *n*-butyrate were influenced by diet (P < 0.001); the acetate decreased and the *n*-butyrate increased during fermentation of inoculated silages compared with GS. The E values ranged from 74.4 to 75.6 molar proportions, and in the inoculated GS they were higher compared with GS (P < 0.05). Compared with GS, the concentration of ammonia N of the inoculated GS substrates was lower (P < 0.01). During fermentation of inoculated GS, the EMS values increased in both the GS + EF2/3s and GS + EF26/42 substrates compared to GS (P < 0.05).

Fatty acid contents and biohydrogenation of grass silages in RUSITEC

The diets (D) affected the daily outputs of myristic, palmitic, stearic, oleic, TVA, linoleic acid, CLA, α-linolenic acid and total FA (Table 3). Fermentation of the inoculated GS resulted in interaction effects of diet and time (D × T) for the outputs of myristic, palmitic, stearic, oleic, TVA, CLA and α-linolenic acid. Compared with the GS, the fermentation of the GS + EF2/3 and GS + EF26/42 lowered the output of linoleic acid (P < 0.05) and total FA (P < 0.05 and P < 0.001). The BH of oleic, linoleic, α-linolenic and total C18 unsaturated FA was not affected (Table 3).

Rumen fermentation parameters of maize silages in RUSITEC

The diet (D) affected the molar proportion of acetate, propionate, *n*-butyrate, A/P ratio, methane, ammonia N, N incorporated by microflora (N_M) and efficiency of microbial protein synthesis (EMS) in maize fermentation in the RUSITEC (Table 4). The acetate and *n*-butyrate increased and propionate decreased during fermentation of inoculated MS silages compared with MS. Compared with MS, the concentration of methane and ammonia N of the inoculated MS substrates was lower (P < 0.05 and P < 0.01). During fermentation of inoculated MS, the EMS and N_M values increased in both the MS + EF2/3s and MS + EF26/42 substrates compared to MS (P < 0.05 and P < 0.01).

Fatty acid contents and biohydrogenation of maize silages in RUSITEC

The diets (D) affected the daily outputs of oleic acid and total FA in maize silages (P < 0.001; Table 5). Compared with the MS, the fermentation of the MS + EF26/42 increased the output of total FA (P < 0.001). The BH of oleic, linoleic, α-linolenic and total C18 unsaturated FA was affected by the diets (P < 0.05, P < 0.01 and P < 0.001). The average apparent BH of these FA was higher in MS + EF26/42 and lower in MS + EF2/3s as compared to control (P < 0.05).

DISCUSSION

The effect of grass silages on rumen fermentation and lipid metabolism in RUSITEC

The inoculants (EF2/3 and EF26/42) were well established

in grass silages (Váradyová et al., 2013). Despite the fact that the dry matter and ADF were lower in inoculated grass silages, the degradability of DM, degraded NDF and degraded ADF of grass silage diets (that is, GS, GS + EF2/3 and GS + EF26/42) were comparable in the RUSITEC experiments. These findings are consistent with our recent results with grass silage inoculants (that is, *L. plantarum* CCM4000, *L. fermentum* LF2 and *E. faecium* CCM4231) in RUSITEC (Jalč et al., 2009c). The losses of DM degradability could be caused by higher fermentation activity connected with the catabolism of cellulose and hemicellulose in inoculated GS. It is known that DM losses up to 12% during ensilage are a useful indicator of inoculant activity in silages (Driehuis et al., 2001). The total SCFA in RUSITEC effluent did not differ among diets. These finding are consistent with prior results in sheep fed GS inoculated with *L. plantarum* + *Pediococcus acidilactici* and in fermentations of GS inoculated with *L. plantarum* CCM4000, *L. fermentum* LF2 and *E. faecium* CCM4231 in RUSITEC (Jatkauskas and Vrotniakiene, 2006; Jalč et al., 2009a).

The silage inoculants of LAB can increase the concentration of SCFA in rumen fluid or do not have any consistent effect on total concentration of SCFA (Weinberg et al., 2004). Effect of diet on molar proportions of acetate and butyrate was observed; the shift could be ascribed to carbohydrate fermentation in RUSITEC. It is known that the composition of the raw material of silages strongly affects the fermentation process. Addition of barley grain balanced diet resulted to the higher energetic efficiency of SCFA's. The use of inoculated (*L. plantarum* + *Streptococcus*, *E. faecalis*) GS in heifers was associated with a change in rumen SCFA pattern, with a higher molar proportion of propionate and a corresponding reduction in both acetate and butyrate (Sharp et al., 1994). In GS diets, more than half of lactic acid from silage is usually converted to propionate, and the remainder is nearly equally divided between acetate and butyrate (Shingfield et al., 2002).

The ammonia concentrations in RUSITEC ranged from 218.27 (GS) to 150.21 (GS + EF2/3s) and 151.47 (GS + EF26/42) mg/l. These reductions might be explained by the greater utilization of ammonia N produced by rumen microorganisms with access to a readily available energy source, increasing microbial protein synthesis or by reduction in the use of amino acids as an energy source by microorganisms (Lee et al., 2003). The maximum rate of digestion in ruminants was observed when ammonia concentration was between 45 to 60 mg/l in forage based diets and between 200 to 270 mg/l in starch based diets (Boniface et al., 1986; Mehrez et al., 1977). Ammonia seems to be sufficiently supplied to rumen bacteria as efficiency of microbial protein synthesis (EMS) ranged from 20.3 to 25.0 mg N/g organic matter fermented (OMF) (ARC, 1984). The EMS values in inoculated GS diets (27.2 to 29.3 mg/g OMF) were near the range of those previously mentioned. In our RUSITEC experiment, the inoculated diets (that is, GS + EF26/42 > GS + EF2/3s) were not effective in increasing TVA and CLA in fermentation fluid. Recent study showed that the inoculant *L. plantarum* CCM4000 added to the grass silage diet was effective in increasing CLA in RUSITEC (Jalč et al., 2009c). It is clear that a decrease in α-linolenic and linoleic acids is accompanied by an increase in stearic acid and the appearances of TVA and CLA in the diets as a result of lipolysis and biohydrogenation (Lee et al., 2003, 2006). When the D × T interaction was significant in lipid metabolism, we can speculate that differences may be caused by differences in microbial populations developed during fermentation in RUSITEC. The net losses of FA across the rumen with grass and clover silage diets as partial results of different microbial population have been reported previously (Lee et al., 2003, 2006). Positive correlation between increasing amounts of linoleic acid in the diets of cows and linoleic acid uptake by ruminal bacteria has been also reported (Bauchart et al., 1990). It is evident that different ruminal bacteria can selectively synthesize FA acids and can participate in the biohydrogenation of FA (Li et al., 2012). Although, the content of α-linolenic and linoleic acid in the inoculated GS was higher than in the uninoculated GS (Table 1), neither of the two inoculated diets (that is, GS + EF2/3 and GS + EF26/42) was effective for decreasing biohydrogenation in RUSITEC. However, some authors suggested that there is no relationship between dietary concentrations of linoleic acid and the level of rumen BH (Doreau and Ferlay, 1994).

In our experiment, the average values of C18:2 FA BH ranged from 93.3 to 96.2% and C18:3 from 98.3 to 98.9% for all GS diets. These results are consistent with the results describing almost complete BH of C18:2 (70 to 95%) and C18:3 (85 to 100%) in the rumen (Doreau and Ferlay, 1994). The average values of BH of C18:2 and C18:3 from 83 to 89% and from 85 to 90% in the rumen of steers fed experimental silages (that is, grass, red clover and white clover) were reported (Lee et al., 2003, 2006).

The effect of maize silages on rumen fermentation and lipid metabolism in RUSITEC

In this study, the counts of inoculants decreased during ensiling and at the end of ensiling (days 111), their counts were about 1 to 1.6 log10 cfu/g in maize silages. In addition, *Enterococcus* spp. levels decreased in maize silage after 3 weeks ensiling period from 7.0 to 4.0 log 10 cfu/g (Masiello, 2010). Despite the fact that in inoculated maize silages (MS + EF26/42, MS + EF2/3s) had higher IVDMD than uninoculated MS, the parameters of rumen fermentation (degradability of DM, NDF and ADF) were comparable in all MS diets during fermentation in RUSITEC.

When inoculated MS diet with bacterial inoculant was fed to steers and inoculated MS with *L. acidophilus* and *Propionibacteria freudenreichii* was fed to cows degradability of DM, OM and NDF were not altered (Silva et al., 2006; Raeth-Knight et al., 2007). Although, no differences were found in the production of total SCFA and individual SCFA's (mmol/day) among the diets; the differences were found in molar proportions (mol%) of acetate, propionate, n-butyrate and A/P in inoculated MS diets compared to control. In addition, no differences in total SCFA production in cows fed a total mixed ration (TMR) containing 462 g/kg of MS and TMR with inoculated MS were found (Raeth-Knight et al., 2007). The silage inoculants (LAB) can increase SCFA production in rumen fluid or do not have any consistent effect on total SCFA concentration (Weinberg et al., 2004). The ammonia concentration ranged from 232.4 (MS) to 154.7 (MS + EF2/3s) and 174.7 (MS + EF26/42) mg/l.

The reduction of rumen ammonia concentration in inoculated MS diets could be explained by greater utilization of ammonia produced by microorganisms with access to a readily energy source increasing microbial protein synthesis (Lee et al., 2003). Ammonia seems to be sufficiently supplied to rumen bacteria as EMS ranged from 20.3 to 25.0 mgN/g OMF (ARC, 1984). The EMS values in inoculated MS diets (20.2 to 24.7 mg N/g OMF) were within the range of those previously mentioned. High variation in EMS has often been described in both *in vivo* and *in vitro* (Armstrong, 1980; Carro and Miller, 1999). In addition, limited studies are available where the effect of inoculated maize silage diets on lipid metabolism was studied *in vitro* (Jalč et al., 2009d). The daily outputs of fatty acids (C18:2 and C18:3; g/100g FA per vessel) were similar in all MS diets, while the outputs of C18:1 in inoculated MS diets were significantly decreased. The outputs of the isomers-*cis9*, *trans*11 C18:2 and *trans* 11 C18:1 were similar during fermentation of all MS diets, with tendency of higher values in MS + EF2/3s diet. In our previous study, when three inoculated maize silage diets (*L. plantarum* CCM 4000, *L. fermentum* LF2, *E. faecium* CCM 4231) were used and fermented in RUSITEC, any of inoculated maize silage diets were effective in increase of CLA and TVA in effluent compared to no-inoculated maize silage diet (Jalč et al., 2009d). The biohydrogenation (BH) of fatty acids (C18:1, C18:2 and C18:3) was different during fermentation of inoculated MS diets.

The decrease of BH in C18:1 and C18:2 was probably connected with increase of CLA and TVA output in MS + EF2/3s diet. Conversely, BH of C18:1, C18:3 and total C18 increased during fermentation of MS + EF26/42 diet. BH values in present experiment for C18:2 and C18:3 were in the range of 0.70 to 0.90 and 0.85 to 1.0, respectively reported previously (Doreau and Ferlay, 1994).

Conclusion

The inoculated GS diets decreased proportion of acetate and increased the proportion of *n*-butyrate and the inoculated MS diets increased proportion of acetate, *n*-butyrate and decreased proportion of propionate, methane and ammonia nitrogen compared to control. The efficiencies of microbial protein synthesis were increased by the inoculated GS and MS diets. Neither of the two inoculated diets (that is, GS + EF2/3 and GS + EF26/42) was effective in increasing TVA and CLA or in decreasing biohydrogenation in RUSITEC effluent. Inoculated maize silages (MS + EF2/3s and MS + EF26/42) had similar effect on rumen fermentation; MS + EF2/3s had positive effect on BH as compared to control diet. In future studies, ensiling conditions must be modified for maintaining an inoculant population in MS (short time of ensiling, the use of inoculants other as enterococci).

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