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Effects of microbial inoculants *Enterococcus faecium* EF2/3s and EF26/42 on microbial, chemical, and fermentation parameters in grass silage

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Abstract: Survival of inoculants in grass silages may enable them to improve the quality of silages through enrichment with polyunsaturated fatty acids (PUFAs). The effects of 2 inoculants, *Enterococcus faecium* 2/3s (EF2/3s) and *E. faecium* 26/42 (EF26/42), on nutrient composition, fermentation parameters, and fatty acid content in grass silages during ensiling (111 days) of fresh grass (G) were examined under laboratory conditions. The G [285 g of dry matter (DM) kg^{-1}] was ensiled in 36 polyethylene jars (1 L) divided into 3 × 12 sets per treatment and ensiled at 21 °C for 111 days. The 3 silage treatments used were: 1) grass without inoculant (GS, control), 2) grass inoculated by the strain EF2/3s (GS+EF2/3s), and 3) grass inoculated by the strain EF26/42 (GS+EF26/42). The inoculant strains were sufficiently established during ensiling and reached 4.62 log_{10} cfu g^{-1} for EF2/3s and 3.76 log_{10} cfu g^{-1} for EF26/42 on day 111 of ensiling. Crude protein contents were G > GS+EF2/3s > GS+EF26/42 > GS (129, 110, 109, and 100 g kg^{-1} of DM, respectively). The lactate-to-acetate ratios were GS < GS+EF26/42 < GS+EF2/3s (2.82, 4.20, and 4.70, respectively). Concentrations of α-linolenic acid and γ-linolenic acid were highest in grass before ensiling ($P < 0.001$). Higher isomer C18:2 (9,11) content ($P < 0.01$) was detected in GS+EF2/3s and GS+EF26/42 than in GS. Nutritional manipulation associated with *Enterococcus faecium* EF2/3s and EF26/42 inoculation of GS resulted in better quality of silages based on lower lactate (GS+EF26/42) and a greater lactic-to-acetic acid ratio (GS+EF2/3s and GS+EF26/42). This might positively affect PUFAs and their isomers.

Key words: Degradation, fatty acids, fermentation biotechnology, grass silage, lactic acid bacteria

1. Introduction

Fresh grass dry matter (DM) contains 1%–3% fatty acids (FAs), and FAs themselves contain 50%–75% α-linolenic acid and 5%–15% linoleic acid (Schroeder et al. 2004). When forages are conserved as silage directly after cutting or after wilting for a short time (<24 h), the concentration of FAs remains relative stable (Arvidsson et al. 2009). Microbial inoculants are applied to forage at the time of ensiling to accelerate a drop in pH during the initial stage of silage fermentation, thus preserving plant carbohydrates and plant proteins by decreasing proteolysis and deamination (Seale 1986). Silage inoculants have advantages over chemical additives; they are easy to apply, do not pollute the environment, and are regarded as natural substances. Some in vitro experiments have shown that microbiota such as lactobacilli, lactococci, propionibacteria, bifidobacteria, and enterococci are able to form conjugated linoleic acids (CLA; *cis*9 *trans*11 C18:2) from linoleic acid (LA) in a growth medium (Coakley et al. 2003; Sieber et al. 2004). Based on previous screenings at our institute (Marciňáková 2006), some strains of lactobacilli and enterococci isolated from rumen fluid and silages were able to convert LA to CLA under in vitro conditions. Previously, 3 of the target isolates (*Enterococcus faecium* CCM4231, *Lactobacillus plantarum* CCM4000, *Lactobacillus fermentum* LF2) were used as inoculants for the ensiling of grass (Jalč et al. 2009a). Studies using only enterococci as silage inoculants are limited (Marciňáková et al. 2008; Jalč et al. 2009a, 2009b). Therefore, in the present study, the other 2 species (*Enterococcus faecium* EF2/3s and EF26/42) from this screening were used to determine the effect of these inoculants on microbial status, chemical parameters [including polyunsaturated fatty acids (PUFAs)], and fermentation parameters in grass under the silage process.

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2. Materials and methods

2.1. Treatments and experimental design

A total area of 6 ha of first-cut orchardgrass (*Dactylis glomerata*) was cut on 1 June 2011 (at the early bloom stage of maturity), spread to cover the total harvested area, and wilted for 16 h. After wilting, the grass was chopped to a length of 20 mm with a forage chopper. Three silage treatments were used for the ensiling: untreated grass silage (GS, control) without inoculants, treated grass inoculated with the strain *E. faecium* EF2/3s (GS+EF2/3s), and treated grass inoculated with the strain *E. faecium* EF26/42 (GS+EF26/42). Approximately 50 kg of grass was mixed in a plastic container to consolidate the herbages. For each silage treatment, approximately 15 kg of mixed grass was used for ensiling. The diluted inoculants were sprayed at 10 mL kg grass^{-1}. The control was sprayed with 10 mL of distilled water kg grass^{-1}. For ensiling, a fresh culture of each inoculant strain was diluted with Ringer solution to a population of at least 10^9 cfu mL^{-1}. About 900 g of grass was then pressed into 1-L polyethylene (PET) jars, with 36 PET jars divided into 3 × 12 sets per treatment. The PET jars were sealed with lids, and the joints were filled with paraffin to prevent the inlet of oxygen. Ensiling of the grass took place at 17–21 °C for 111 days in a dark room. Representative samples of grass were collected from 5 different places of 50 kg of grass, and average samples (in triplicate) were used for microbiological and chemical analyses before division into PET jars (days 0–1). In addition, 2 PET jars per treatment were opened on days 7, 40, and 111 of ensiling for microbiological analyses (in triplicate), and 2 other PET jars per treatment were opened for nutritional analyses and fermentation parameters (in triplicate) after ensiling. After sample collection, the jars were discarded. Thus, from 12 jars per treatment the last 4 PET jars were stored and used for future in vitro rumen experiments.

2.2. Microbiological analyses

Both silage inoculants (*E. faecium* 2/3s and *E. faecium* 26/42) were isolated and characterised at our institute (Laboratory of Animal Microbiology). To inoculate silage by rifampicin (Sigma, Deisenhofen, Germany), marked *E. faecium* EF2/3s and EF26/42 were used to differentiate them from total enterococci (Strompfová et al. 2003). To enumerate the silage inoculants, enterococci, amylolytic streptococci and other lactic acid bacteria (LAB), and *Listeria*-like bacteria, the samples were treated according to the standard microbiological dilution method (International Organization for Standardization); 10 g of ensiling grass was mixed with 90 mL of Ringer solution (pH 7.0; Oxoid, Basingstoke, England) treated by a stomacher (IUL Masticator, Barcelona, Spain), and then 100 µL aliquots of serial dilutions were plated onto m-Enterococcus agar (Difco, Becton and Dickinson, Cockeysville, MD, USA) to enumerate enterococci and onto m-Enterococcus agar enriched with rifampicin (100 µg mL^{-1}) to differentiate the inoculants from common enterococcal flora. MRS agar (de Man–Rogosa–Sharpe agar, Merck, Darmstadt, Germany) was used to detect other LAB. Amylolytic streptococci were isolated on M17 agar (Oxoid) supplemented with maize starch, and *Listeria*-like bacteria were isolated on McBridge agar (Becton and Dickinson). Inoculant counts were expressed in colony-forming units ($\log_{10}$ cfu) per gram ± standard deviation.

2.3. Chemical analysis

Chemical analysis was performed in triplicate and is presented on a DM basis. Samples of fresh grass and grass silages were determined by oven drying at 65 °C for 48 h for DM, neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) using a Fibertec 2010 (Tecator Comp., Höganäs, Sweden) (Van Soest et al. 1991). ADF is expressed inclusive of residual ash. NDF was assayed with sodium sulphite and expressed inclusive of residual ash (Mertens 2002). Standard methods (AOAC, 1990) were used to determine the ash (AOAC Official Method No. 942.05), nitrogen (AOAC Official Method No. 968.06), fat (AOAC Official Method No. 983.23), and crude protein (AOAC Official Method No. 990.03; AOAC, 1990). In vitro dry matter degradability (IVDMD) of the fresh grass and grass silages was determined from the difference between the substrate weight before and after the 72-h incubation of batch cultures with rumen inocula, according to the methods described by Váradyová et al. (2005). Rumen inoculum for IVDMD was obtained from 3 rumen-cannulated Slovak Merino sheep (aged 7 years, mean body weight 43 ± 2.4 kg) and collected 3 h after the morning feeding into a prewarmed (39 ± 0.5 °C) collection vessel filled with CO_2. A water extract of silages was prepared by adding deionised water (50 mL) to 10 g of silage. The water extract was measured for pH (pH meter, Inolab, level 1, Weilheim, Germany), lactic acid, volatile fatty acids (VFAs), and ammonia nitrogen (AOAC Official Method No. 920.0; AOAC, 1990). Lactic acid as well as acetic, propionic, and n-butyric acids were analysed using the single-column ITP analyser unit Ionosep 2003 (Recman Laboratory Equipment, Ostrava, Czech Republic). The device is a fully automated isotachophoregraph controlled by a computer. Fatty acids in the fresh grass and grass silages were determined in lyophilised samples. Samples of grass and grass silages were freeze-dried using a Thermo Savant MicroModulyo freeze-drier (Thermo Savant MicroModulyo, Holbrook, NY, USA), placed in precleaned high-density polyethylene flasks, and kept in the dark at laboratory temperature until analysed. Lipids from the freeze-dried grass and grass silages were extracted from 0.5 g of the sample with a 2:1 mixture of chloroform and

methanol; samples were purified using 20% HCl (Bligh and Dyer 1959). The extracted lipids were dissolved in 1 mL of hexane and 1 mL of internal standard (tridecanoic acid; Fluka, Chemie GmbH, Buchs, Switzerland). Subsequently, 2 mL of transesterification reagent (1 N methanolic sodium methoxide; Fluka) 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 FA methyl esters by gas chromatography. Samples were injected by splitless injector into a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer, Inc., Shelton, CT, USA) equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm; Agilent Technologies, Inc., Santa Clara, CA, USA) and flame ionisation detector (constant flow, hydrogen 40 mL min^{-1}, air 400 mL, 260 °C). Analyses of FAs (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; 130–170 °C at program rate 6.5 °C min^{-1}; 170–206 °C at program rate 1 °C min^{-1}; 206–240 °C at program rate 34 °C min^{-1}) with hydrogen as the carrier gas (flow 1.8 mL min^{-1}, velocity 44 cm s^{-1}, pressure 23.2 psi). The FA methyl ester peaks were identified with a commercial mixture (Supelco 37 Component FAME MIX, Supelco; *trans*11-vaccenic methyl ester, Supelco; *cis*9 *trans*11 conjugated linoleic acid, Matreya, Pleasant Gap, PA, USA) and quantified by the internal standard of tridecanoic acid (C13:0; Supelco).

2.4. Statistical analysis

Statistical analysis was performed using analysis of variance (GraphPad InStat, GraphPad Software Inc., San Diego, CA, USA). The experiment was completely randomised in design with 3 replicates per treatment. The results were analysed by one-way analysis of variance. When the overall treatment effect was significant ($P < 0.05$), individual treatment differences were determined using the Bonferroni multiple comparison test. Significance was set at $P < 0.05$.

3. Results

3.1. Microbiological parameters of silages

The inoculant strains were sufficiently established during ensiling (Table 1). The counts of inoculants reached a maximum on days 7 and 40 of ensiling (7.76 or 7.49 for EF2/3s and 7.96 or 8.08 log_{10} cfu g^{-1} for EF26/42, respectively). On day 111 of ensiling, the counts of inoculants reached 4.62 log_{10} cfu g^{-1} for EF2/3s and 3.76 log_{10} cfu g^{-1} for EF26/42. The counts of *Listeria*-like bacteria in inoculated silages decreased numerically when compared with GS.

Table 1. Bacterial counts (log_{10} cfu g^{-1}) in grass silages during ensiling (n = 3).

Grass silages (GS)	EF2/3s, EF26/42	Other LAB	*Enterococci*	Amylolytic *streptococci*	*Listeria*-like bacteria
Days 0–1					
GS	No growth	3.68 ± 0.92	3.95 ± 0.98	6.47 ± 0.54	4.80 ± 0.19
GS+EF2/3s	4.90 ± 0.21	4.76 ± 0.18	4.72 ± 0.17	5.33 ± 0.30	4.08 ± 0.20
GS+EF26/42	4.83 ± 0.17	4.71 ± 0.17	5.46 ± 0.33	5.74 ± 0.39	4.55 ± 0.13
Day 7					
GS	No growth	7.24 ± 2.69	7.46 ± 2.73	7.79 ± 2.79	5.89 ± 2.42
GS+EF2/3s	7.76 ± 2.78	7.90 ± 2.81	7.71 ± 2.77	7.99 ± 2.82	5.07 ± 2.25
GS+EF26/42	7.96 ± 2.82	7.89 ± 2.79	7.81 ± 2.79	7.95 ± 2.81	5.64 ± 2.37
Day 40					
GS	No growth	8.58 ± 2.93	7.25 ± 2.69	9.76 ± 3.12	5.23 ± 2.28
GS+EF2/3s	7.49 ± 2.73	7.55 ± 2.74	7.56 ± 2.75	9.48 ± 3.07	4.82 ± 2.19
GS+EF26/42	8.08 ± 2.84	7.87 ± 2.80	8.50 ± 2.93	8.70 ± 2.96	4.86 ± 2.20
Day 111					
GS	No growth	6.51 ± 2.55	6.75 ± 2.59	6.30 ± 2.10	6.64 ± 2.57
GS+EF2/3s	4.62 ± 2.14	6.20 ± 2.48	6.04 ± 2.50	6.82 ± 2.61	3.85 ± 1.94
GS+EF26/42	3.76 ± 1.94	7.45 ± 2.72	6.58 ± 2.56	7.33 ± 2.70	3.90 ± 2.01

LAB = lactic acid bacteria; EF2/3s = *Enterococcus faecium* EF2/3s; EF26/42 = *Enterococcus faecium* EF26/42. Values are means ± standard deviations.

3.2. Nutrient composition and fermentation parameters in grass silages

The mean DM content in grass before ensiling (G) was 285 g kg^{-1}, and ensiling resulted in a significant decrease ($P < 0.001$) in the DM content of inoculated GS compared with G and GS (Table 2). The crude protein (CP) content was G > GS+EF2/3s > GS+EF26/42 > GS (129, 110, 109, and 100 g kg^{-1} of DM, respectively). The value of NDF was the lowest in GS+EF2/3s ($P < 0.001$). The ADF content was GS > GS+EF2/3s > GS+EF26/42 > G (431, 410, 376, and 337 g kg^{-1} of DM, respectively). The values of N, ADL, fat, ash, and pH did not differ across treatments. In comparison to GS, IVDMD showed lower values in G and in GS+EF26/42 ($P < 0.001$). Inoculated GS+EF26/42 contained lower values for lactate ($P < 0.001$); both inoculated GS (GS+EF2/3s and GS+EF26/42) contained lower values for acetate ($P < 0.001$) and propionate ($P < 0.001$) compared with GS. The lactate-to-acetate ratio of inoculated GS was greater ($P < 0.01$) than in GS. N-butyrate was not detected in silages. The ammonia N of GS+EF2/3s decreased ($P < 0.01$) when compared with GS.

3.3. Fatty acids composition of grass silages

The content of palmitic acid in G and inoculated GS was higher ($P < 0.01$) in comparison with GS (Table 3). The stearic acid concentrations were G > GS+EF2/3s > GS+EF26/42 > GS (50.2, 42.7, 23.3, and 21.5 g kg^{-1} of FAs, respectively). The content of *cis* C18:2n-6 LA in GS+EF26/42 was the highest ($P < 0.001$). Concentrations of α-linolenic acid and γ-linolenic acid were highest in grass before ensiling ($P < 0.001$). The content of isomer C18:2 (9,11) in GS+EF2/3s and GS+EF26/42 was higher ($P < 0.001$) when compared with the GS and G. Concentrations of total C18 FAs were G > GS+EF26/42 > GS+EF2/3s > GS (683, 574, 552, and 482 g kg^{-1} FA, respectively). The contents of saturated FA in the grass and GS+EF2/3s were higher ($P < 0.001$) compared with GS ($P < 0.01$), whereas the concentrations of unsaturated FA were lower ($P < 0.05$). The contents of short-chain FA in GS+EF26/42 and medium-chain FA in GS were lower across all treatments.

Table 2. Nutrient composition in grass before ensiling (G) and grass silages (GS, GS+EF2/3s, and GS+EF26/42) after 111 days of ensiling (n = 3).

	G	GS	GS+EF2/3s	GS+EF26/42	SEM	P-value
DM (g kg^{-1})	285a	285a	272b	279c	8.5	***
Nitrogen (g kg^{-1} DM)	20.6	16.0	17.5	17.5	6.51	Ns
Crude protein (g kg^{-1} DM)	129c	100a	110b	109b	15.9	***
NDF (g kg^{-1} DM)	663a	670a	607b	682a	59.4	***
ADF (g kg^{-1} DM)	337d	431a	410b	376c	36.7	***
ADL (g kg^{-1} DM)	46.3	49.1	59.1	61.5	14.68	Ns
Fat (g kg^{-1} DM)	21.8	31.6	29.3	28.4	2.14	Ns
Ash (g kg^{-1} DM)	60.3	55.9	59.4	56.6	12.62	Ns
IVDMD (g kg^{-1} DM)	475c	542a	522a	438bc	10.1	***
pH	-	4.47	4.29	4.21	0.422	Ns
Lactate (g kg^{-1} DM)	-	72.1a	78.7a	57.0b	5.31	***
Acetate (g kg^{-1} DM)	-	25.6a	16.7b	13.6c	0.31	***
Lactate/acetate	-	2.82a	4.70b	4.20b	0.32	**
Propionate (g kg^{-1} DM)	-	16.3a	11.4b	10.4c	0.21	***
NH_3 N (mg N 100 g^{-1} DM)	-	83.1a	56.7b	86.7a	5.42	**

EF2/3s = *Enterococcus faecium* EF2/3s; EF26/42 = *Enterococcus faecium* EF26/42; DM = dry matter; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; IVDMD = in vitro dry matter degradability.
Ns = not significant at $P > 0.05$; overall model P-value at **$P < 0.01$, ***$P < 0.001$.
[a–d]: Within a row, means without a common superscript letter differ at $P < 0.05$.

Table 3. Fatty acid (FA) composition (g kg^{-1} FA) of grass before ensiling (G) and grass silages (GS, GS+EF2/3s, and GS+EF26/42) after 111 days of ensiling (n = 3).

	G	GS	GS+EF2/3s	GS+EF26/42	SEM	P-value
C10:0 caprinic	3.60	2.65	3.29	2.32	0.371	Ns
C12:0 lauric	16.7	13.0	16.0	11.7	1.04	Ns
C14:0 myristic	16.4	14.8	14.0	13.2	1.42	Ns
C16:0 palmitic	211b	177a	231b	221b	10.5	**
C18:0 stearic	50.2b	21.5a	42.7b	23.3ac	3.37	***
cis C18:1n-9 oleic	30.1	33.8	43.7	48.5	3.92	**
cis C18:2n-6 linoleic	121a	112a	120a	181b	6.5	***
C18:3n-3 α-linolenic	477b	314a	345a	320a	12.1	***
C18:3n-6 γ-linolenic	4.47b	0.32a	0.53a	0.08a	0.121	***
Isomer C18:2 (9,11)	0.0	0.0	0.53a	1.14b	0.054	***
Total C18 FA	683c	482a	552b	574b	10.5	***
Saturated FA	325c	248a	327bc	283abc	9.4	***
Unsaturated FA	675c	747a	670bc	717abc	14.1	**
Short-chain FA	22a	19ab	22a	16b	1.1	**
Medium-chain FA	260b	214a	269b	253b	7.6	***
Long-chain FA	718	763	706	730	13.3	Ns

EF2/3s = *Enterococcus faecium* EF2/3s; EF26/42 = *Enterococcus faecium* EF26/42.
Ns = not significant at $P > 0.05$; overall model P-value at $^{**}P < 0.01$, $^{***}P < 0.001$.
$^{a–c}$: Within a row, means without a common superscript letter differ at $P < 0.05$.

4. Discussion

The inoculant *E. faecium* strains were sufficiently established during ensiling. During ensiling, the counts of LAB, enterococci, and amylolytic streptococci were not affected. Bacteriocins (usually called enterocins) produced by enterococci, especially those produced by the species *E. faecium*, possess predominant antilisterial activity. Because both of our inoculants produce bacteriocins, although counts of the *Listeria*-like bacteria of inoculated GS decreased only numerically, the decrease can be explained by the antimicrobial activity of their enterocins (Marciňáková et al. 2004; Lauková et al. 2008; Marciňáková et al. 2008).

In our results, the losses of DM were probably caused by higher fermentation activity connected with catabolism of cellulose and hemicellulose in inoculated GS. DM losses of up to 12% during ensilage are a useful indicator of the activity of inoculants in silages (Driehuis et al. 2001). The crude protein (CP) of the inoculated GS increased when compared with uninoculated GS and reached an optimum range for grass silage, i.e. from approximately 100 to 200 g kg^{-1} DM (Merry et al. 2000). We can speculate that the decrease of NDF in GS+EF2/3s and ADF in both inoculated GS samples (GS+EF2/3s and GS+EF26/42) is due to partial hydrolysis of cellulose. This is in contrast to studies reporting a lack of effect of inoculated treatments on fibre degradability of alfalfa and corn silages (Contreras-Govea et al. 2011). The lower values of IVDMD in GS+EF26/42 are consistent with our previous results, in which the IVDMD of silages inoculated with *L. plantarum* CCM4000 and *L. fermentum* LF2 was lower compared to silage without inoculants (Jalč et al. 2009a). In addition, the effect of 14 microbial inoculants in alfalfa silage showed that 48 h of IVDMD was not improved by inoculation with LAB (Filya et al. 2007).

Regarding the fermentation profile, less lactic, acetic, and propionic acid was found in the inoculated silage (GS+EF26/42), which is consistent with the optimal pH values. These VFAs, as well as n-butyric acid, are usually responsible for most of the drop in silage pH. Inoculants are less effective at improving fermentation in corn silage (40% of cases) than in alfalfa (75%) or grass silages (71%;

Muck et al. 2007). However, in good silage, lactic acid should represent from at least 65% to 70% of the total silage acids. It seems that the application of probiotic and probio-enzymatic commercial bacterial inoculants results in a decrease in pH, increases the lactic acid content, and minimises n-butyric acid content (Skládanka et al. 2012). Our findings (negligible propionate and absence of n-butyrate) are consistent with recent studies of alfalfa silage ensiled with *L. plantarum* MTD/1 or chopped lucerne silages ensiled with *L. plantarum* + *E. faecium* or *L. plantarum* alone (McAllister et al. 1998; Mohammed et al. 2012). The ratio of lactic acid to acetic acid is a good indicator of the efficiency of silage fermentation (Weinberg and Muck 1996). Despite the fact that GS inoculants increased the lactic acid-to-acetic acid ratio in our results, it was still not below 3:1. It is clear that in terms of total silage acids (lactate, acetate, propionate) the inoculated GS contained a higher proportion of lactic acid (73.7% or 70.4% for GS+EF2/3s and GS+EF26/42, respectively) than the uninoculated GS (63.9%). A similar effect in regard to cellulase by itself or in combination with the LAB inoculants *L. plantarum* and *Pediococcus cerevisiae* on orchardgrass silage acids was previously reported (Nadeau et al. 2000).

In the present experiment, the ammonia N in GS+EF2/3s was reduced; however, our previous results showed that inoculated (*E. faecium* CCM4231) grass silages can increase ammonia N concentration in comparison with uninoculated GS (Jalč et al. 2009a). These finding are consistent with results that report LAB strains as grass silage inoculants with the ability to limit the formation of ammonia N (Saarisalo et al. 2007). In addition, LAB also survive in ruminal fluid, directly affect ruminal fermentation, and improve ruminal microbial biomass production through the inoculant effect on silage ammonia N (Weinberg et al. 2003, 2004).

With respect to the FA content in GS, it seems that factors such as rate and extent of fermentation influenced the degree of lipolysis in silages (Khan et al. 2009; Van Ranst et al. 2009). The increase of total C18 FA content (Table 3) in inoculated grass silages may be explained by the concentration of lipid matter due to loss of DM content. In addition, the PUFA ratio of n-3 to n-6 (long chain n-3/total n-6 PUFA ratio) of GS was the lowest in GS+EF26/42, which may indicate better silage in terms of PUFA. The ensiling of forage can reduce the contents of PUFA (Dewhurst and King 1998). This is in contrast to the ensiling of grass rich in C18:3n-3 FA, which does not reduce PUFA concentration (Doreau and Poncet 2000; Khan et al. 2012). In this study the presence of isomer C18:2 (9,11) in inoculated GS was an indication of biohydrogenation activities in silages. The occurrence of isomer C18:2 (9,11) in inoculated GS was likely due to the biohydrogenation pathways of C18:2, which are similar to those that occur by microbial action in the rumen. In GS+EF26/42, the content of isomer C18:2 (9,11) was 2 times higher than in GS+EF2/3s. This is probably due to the higher LA content in GS+EF26/42. The presence of the *trans*11, *cis*15 and *cis*9, *cis*15 C18:2 isomers in grass silages and *trans* C18:1 isomers in grass and red clover silages has been reported in recent studies (Vanhatalo et al. 2007; Alves et al. 2011). Lactic acid bacteria commonly found in forages have the ability to biohydrogenate C18:2n-6 and C18:3n-3, isomerise linoleic acid (C18:2n-6) into conjugated linoleic acid, and reduce the conjugated linoleic acid into *trans*10 C18:1 (Ogawa et al. 2005; Kishino et al. 2009). It is known that strains belonging to the genera *Enterococcus*, *Pediococcus*, *Propionibacterium*, and *Lactobacillus* produce considerable amounts of 2 conjugated linoleic acid isomers (*cis*9, *trans*11 C18:2 and *trans*9, *trans*11 C18:2) (Ogawa et al. 2001). Our results show that both silage inoculants (*E. faecium* 2/3s and *E. faecium* 26/42) that were selected through our screening can produce isomer C18:2 (9,11) under optimal silage conditions. It seems that isomerisation and hydrogenation may occur during ensiling (Alves et al. 2011).

From the results of the present study it can be concluded that both of the silage inoculants (*E. faecium* 2/3s and *E. faecium* 26/42) isolated in our laboratory were well established in the grass silage and were useful in improving both silage quality and the end products of fermentation. Results showed that inoculated grass silages were of better quality than uninoculated silages based on a greater lactic-to-acetic acid ratio and the antibacterial effect of the inoculants on *Listeria*-like bacteria. Both inoculants (*E. faecium* 2/3s and *E. faecium* 26/42) are promising biocatalysts for isomer C18:2 (9,11). It is important to emphasise that future experiments for increasing PUFA in fermentation fluid and milk with inoculated grass silages are necessary both in vitro (RUSITEC) and in vivo (lactating cows). In addition, due to climate change, it will be necessary to test other fodders as silages (Glamočlija et al. 2011).

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