The use of bacterial inoculants for grass silage: their effects on nutrient composition and fermentation parameters in grass silages

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ABSTRACT: The effect of three microbial inoculants (*Lactobacillus plantarum* CCM 4000, *L. fermentum* LF2, and *Enterococcus faecium* CCM 4231) on the fermentation and nutritive value of orchard grass silage was studied under laboratory conditions. The first-cut orchard grass (280 g of dry matter/kg) was ensiled at 21°C for 105 days. All inoculants were applied at 1.0 × 10(9) CFU/ml. Uninoculated silage served as control. After inoculation, the chopped orchard grass was ensiled in 40 (1 l) plastic jars divided into four groups. The counts of the silage inoculants dominated on day 21 of ensiling: CCM 4231 strain amounted to 9.40 ± 0.30 (log10) CFU/g followed by LF2 (8.69 ± 0.39 CFU/g) and by CCM 4000 (7.55 ± 0.39 CFU/g). However, on day 105 (the end of ensiling) the highest counts of *L. plantarum* CCM 4000 were determined. Overall, microbial inoculants generally had a positive effect on orchard grass silage characteristics in terms of lower pH and higher lactic acid concentration. The inoculants significantly increased the lactic to acetic acid ratio in inoculated silages. The total concentration of acids (acetic, propionic, n-butyric, lactic acid) was 2–3 times higher in inoculated silages compared to control silage. The percentage proportion of fatty acids – SFA, UFA, SCFA and MCFA – was similar in all grass silages. Only the proportions of LCFA – α- linolenic acid ($C_{18:3}$) were lower ($P < 0.001$) while those of oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) were higher ($P < 0.001$) in inoculated silages in comparison with control silage.

Keywords: orchard grass; silage; bacterial inoculants; quality

The main aim of silage making is to conserve the plants with a minimum 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 fermentation quality of silages has a major effect on feed intake, nutrient utilization and milk production in ruminants (Huhtanen et al., 2002, 2003). Fresh grass dry matter (DM) contains 1–3% of fatty acids (FA) and about 50–75% of these FA are $C_{18:3}$ (α-linolenic acid – ALA) and 5–15% $C_{18:2}$ (linoleic acid – LA; Schroeder et al., 2004). Concentrations of ALA vary from crop to crop, and with environmental factors such as stage of maturity, genetic differences, as well as season and light intensity (Elgersma et al., 2006). When forages are conserved as silage, they maintain the same concentration of long-chain FA when they are harvested fresh (Chilliard et al., 2001). In contrast, the preservation of herbage by ensiling lowers the concentration of both the total and polyunsaturated fatty acids in dry matter (Dewhurst et al., 2003). Inoculants that contain lactic acid bacteria (LAB) are often used as silage additives to enhance the

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lactic acid fermentation, hence, to better preserve the ensiled material. Numerous papers reported the ensiling of grass with inoculants *Lactobacillus plantarum* (Rooke et al., 1988; Saarisalo et al., 2004; Muck et al., 2007), *Lactobacillus buchneri* (Driehuis et al., 2001) *Lactococcus lactis, Lactobacillus pentosus* (Muck et al., 2007), *Lactobacillus buchneri* (Tyrolová and Výborná, 2008) as well as *Enterococcus faecium* EF9296 (Marciňáková et al., 2008). Bacterial inoculants have advantages over chemical additives because they are easy to use, safe, they do not pollute the environment and are regarded as natural products. Most commercially available inoculants contain homofermentative LAB, which are expeditious and efficient producers of lactic acid and thus improve the silage fermentation. Among the homofermentative LAB the most frequently used are *Lactobacillus plantarum, L. acidophilus, Enterococcus faecium, Pediococcus acidilactici*. Some *in vitro* experiments showed that certain microorganisms – *lactobacilli, lactococci, propionibacteriae, bifidobacteriae* and *enterococci* are able to form conjugated linoleic acid (CLA – *cis* 9, *trans* 11 $C_{18:2}$) from linoleic acid in a special growth medium (Coakley et al., 2003; Sieber et al., 2004). However, Bessa et al. (2000) revealed the possibility of an alternative pathway in the production of CLA from ALA due to extreme microbial diversity in the reticulo-rumen. Our screening of microorganisms also showed that some *lactobacilli* and *enterococci* isolated from rumen fluid and silages were able to convert linoleic acid to CLA in a special growth medium *in vitro* (Marciňáková, 2006). The objectives of this study were to evaluate the effect of three different inoculants (the isolates of our Institute and the strain CCM 4000 was supplied to us by Dr. Nemcová, University of Veterinary Medicine, Košice, Slovakia) during the ensiling (105 days) of fresh grass on fermentation and chemical composition (including mainly polyunsaturated fatty acids – PUFA) as well as microbiological parameters in grass silages. The population of inoculants and selected microbiota in grass silages was also studied.

MATERIAL AND METHODS

Treatments, material and ensiling

The silages were made from first-cut orchard grass (*Dactylis glomerata)* wilted for 16 h. The grass was chopped to a length of the cut of 20 mm with a forage chopper and about 900 g of fresh grass was pressed into 1 l sealed polyethylene jars. The grass dry matter (DM) was 279.7 g/kg DM and it contained crude protein 150 and NDF 515.5 g/kg DM. The ensiling of grass was carried out in 40 PET jars which were divided into four groups. The four treatments (each with 10 jars) were used: (1) untreated grass (control) without inoculant; (2) grass inoculated by the strain CCM 4231 of *Enterococcus faecium*; (3) grass inoculated by the strain LF2 of *Lactobacillus fermentum*; (4) grass inoculated by the strain CCM 4000 of *L. plantarum*. For the ensiling experiments a fresh culture of each inoculant strain was diluted with Ringer solution to a population of 10^9 CFU/ml. The diluted culture was applied at 10 ml per kg grass. The ensiling of grass was carried out at 21°C for 105 days. Representative samples of the raw material (untreated chopped grass) were taken for microbiological and chemical analyses before the division into jars. In addition, two jars per treatment were opened on days 7, 21, 40 and four jars on 105 day of ensiling for microbiological and chemical analyses.

Characterization of silage inoculants

Enterococcus faecium CCM 4231 (Lauková et al., 1993) is the first described bacteriocin-producing isolate of rumen origin possessing probiotic properties which is able to transform linoleic acid into CLA (Marciňáková, 2006). *Lactobacillus plantarum* CCM 4000 is a ruminal isolate (Nemcová, 1989) possessing probiotic properties and also having the ability to transform LA into CLA. *L. fermentum* LF2 is an isolate from canine feed (Marciňáková, 2006) having good adhesive capability to IPEC-J2 cells.

Microbiological analyses

To determine the counts of the inoculants as well as of the other enterococci and lactic acid bacteria 10 g of ensiling grass and/or silage was sampled and mixed with 90 ml of Ringer solution (pH 7.0, Basingstoke, England); 100 µl aliquots of serial dilutions were plated into the following media (in duplicate): M-Enterococcus agar to enumerate enterococci, MRS (De Man-Rogosa-Sharpe) agar to enumerate LAB (Becton and Dickinson, Cockeysville, USA; Merck, Darmstadt, Germany).

Table 1. Nutrient composition of grass (orchard grass, *Dactylis glomerata*) before ensiling

Dry matter (g/kg)	Crude protein	Crude fibre	NDF	ADF (g/kg/DM)	Lignin	Fat	Ash	IVDMD (%)
279.7	149.7	415.2	515.5	316.4	30.1	25.1	72.5	71.4

NDF – neutral detergent fibre; ADF – acid detergent fibre; IVDMD – *in vitro* dry matter digestibility

To differentiate *E. faecium* CCM4231 strain from the other enterococci, its rifampicin-marked variant was prepared by the subsequent cultivation of the strain using Todd-Hewitt agar (Becton and Dickinson) with rifampicin (100 µg/ml) at 37°C according to Strompfová (2004). To differentiate *L. plantarum* CCM 4000 as well as *L. fermentum* LF2 among the counts of lactic acid producing bacteria the strains were also marked by rifampicin. Bacterial counts were expressed in colony forming units (log10 CFU) per ml per g.

Chemical analyses

Grass dry matter was determined by oven drying at 103°C for 16 hours. Dried (60°C, 48 h) samples were analysed for neutral detergent fibre NDF, acid detergent fibre ADF and lignin (Goering and Van Soest, 1970) using Fibertec 2010 (Tecator Comp., Sweden). Standard methods were used for determining ash (AOAC, 1990), nitrogen (AOAC, 1990), fat (AOAC, No. 983.23) and crude fibre (AOAC, 1990). *In vitro* dry matter digestibility (IVDMD) of grass and grass silages was determined by the *in vitro* fermentation gas production method (Váradyová et al., 2005). A water extract of silage was prepared by adding deionized water to 20 g of silage to obtain a total amount of 300 g. The water extract was measured for pH, organic acids and ammonia nitrogen (AOAC, No. 920 03). Organic acids – lactic acid and volatile fatty acids (VFA: acetic, propionic, n-butyric acid) were analysed on Ionosep 2003. The fatty acids in grass and grass silages were determined in lyophilized samples. Lipids from freeze-dried grass and grass silages were extracted using an extraction – transesterification procedure described by Sukhija and Palmquist (1988). A mixture of chloroform and methanol (2:1) was chosen as the extraction solvent. The extracted lipids were dissolved in 1 ml hexane with internal standard $C_{13:0}$ and the esterification of lipids was carried out with 2 ml N sodium methoxide (30 min, 50°C) and 3 ml 3N methanolic HCl (60 min, 50°C). After centrifugation (5 min, 2 500 rpm) the samples as the upper hexane layers were used for the gas chromatographic analyses. The analysis of methyl esters was performed using a GC 6890N Agilent Technology gas chromatograph equipped with a programmed 60 m HP-Innowa capillary column (180–240°C) and FI detector.

Statistical analysis

The means of results from treatments were analysed by one-way ANOVA using the Student-Newman-Keuls test (Graphpad Instat, Graphpad Software Inc., San Diego, CA, USA). Differences between the treatment means were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

The nutrient composition of orchard grass before ensiling is shown in Table 1. The effect of the used inoculants on grass silage characteristics (microbiological, nutritional) was studied. Silage samples were subjected to analyses that included the determination of dry matter, nitrogen, fat, ash, crude fibre, detergent fibre – NDF, ADF. In addition to the organic acids – lactic, propionic, n-butyric, fatty acids were quantified in this experiment. The mean DM content in grass before ensiling was 279.7 g/kg DM and ensiling resulted in a significant decrease ($P < 0.001$) in the DM concentration of orchard grass. Control silages had 5% and inoculated silages 2–11% lower IVDMD than wilted orchard grass. In comparison with control silage, IVDMD showed significantly ($P < 0.01$) lower values in the silages inoculated with CCM 4231, LF2 or numerically higher values in the silage with the strain CCM 4000 (Table 2). The effect of 14 microbial inocu-

Table 2. Nutrient composition and fermentation parameters in grass silages after 105 days of ensiling (n-4)

	Control	*E. faecium* CCM 4231	*L. fermentum* LF 2	*L. plantarum* CCM 4000	Pooled S.E.M.
DM (g/kg)	222.8	241.5***	229.0*	246.6***	1.8
Ash (g/kg DM)	78.1	77.7	78.0	75.9	1.1
Crude protein (g/kg DM)	126.4	141.5	146.6	139.9	7.6
Crude fibre (g/kg DM)	409.5	365.8*	368.8*	348.3**	9.1
NDF	698.4	686.8	691.3	664.4**	5.1
ADF	407.5	392.2**	385.3**	380.5**	3.2
Fat	24.9	27.3	23.7	24.5*	0.3
IVDMD (%)	66.4	59.9**	60.4**	69.5*	1.0
pH	5.26	4.49***	4.26***	4.35***	0.1
Lactic acid (g/kg DM)	29.6	60.4***	84.9***	94.1***	0.1
Acetate	3.14	1.65***	6.98***	6.89***	0.02
Propionate	–	3.72	4.80	7.70	0.02
Ammonia N (g/kg N)	77.3	92.0***	78.9	55.5***	0.5

DM – dry matter; IVDMD – *in vitro* dry matter degradability; NDF – neutral detergent fibre; ADF – acid detergent fibre; *E. faecium – Enterococcus faecium*; *L. fermentum – Lactobacillus fermentum*; *L. plantarum – Lactobacillus plantarum*
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ differences from the control

lants in lucerne silage showed that 48 h *in vitro* true DM digestibility was not improved by inoculation with lactic acid bacteria (Filya et al., 2007). Crude protein content in all grass silages was significantly decreased compared to grass before ensiling. Also, crude protein content in control silage was numerically (NS) decreased in comparison with inoculated grass silages. Crude protein content varied from 126 to 146 g/kg DM. The optimum minimum mean concentration of grass silage is approximately 160 g/kg DM, although it can range from 39 to 282 g/kg DM (Merry et al., 2000). Compared to the control, crude fibre content as well as detergent fibre (ADF) was significantly or numerically (NDF) lower in all inoculated silages, mostly in CCM 4000 (Table 2). This decrease in detergent fibre content may be due to the partial hydrolysis of cellulose when hemicellulose content in inoculated silages increased. Further results showed that the other parameters of nutrient composition – ash and fat content, respectively, were not affected by the bacterial inoculation during ensiling. The inoculation of ensiled grass influenced the fermentation parameters of grass silage. The mean pH values (about pH 6.0) before ensiling decreased during ensiling and treated silages had significantly lower pH than untreated silage (Table 2). The pH of inoculated silages after 105 days of ensiling tended to be below 4.5, which is considered acceptable for grass silages (Cherney et al., 2006). Lactic acid and other organic acids – acetic, propionic and *n*-butyric acids are usually responsible for the largest part of the drop in silage pH. Lactic acid should account for at least 65% to 70% of the total silage acids in good silage (Shaver, 2003). The lactic acid concentration in silages inoculated with *Enterococcus faecium* CCM 4231, *Lactobacillus fermentum* LF2 and *L. plantarum* CCM 4000 was 2.0, 2.9 and 3.2 times higher ($P < 0.001$) than in untreated silage (Table 2). The concentrations of acetic and propionic acid were low in all grass silages, the concentrations of *n*-butyric acid were not detectable at all. Similar results were reported by McAllister et al. (1998) when two bacterial inoculants (*L. plantarum* + *Enterococcus faecium*) or *L. plantarum* as a single inoculant were used as bacterial inoculants for the ensiling of chopped lucerne. The ratio of lactic acid to acetic acid is a good indicator of the efficiency of silage fermentation. Ideally, the ratio of lactic acid to acetic acid should not be less than 3:1 while the

Table 3. The fatty acid composition of grass silage after 105 days of ensiling (n-4)

Fatty acids (mg/g FA)	Grass silages with inoculants				
	grass silage	*Ent. faecium* CCM 4231	*L. fermentum* LF2	*L. plantarum* CCM 4000	pooled SEM
$C_{8:0}$	0.09	0.66***	1.41***	0.54***	0.005
$C_{10:0}$	8.17	2.60***	3.76***	5.84***	0.05
$C_{12:0}$	3.19	4.66***	5.50***	2.93**	0.05
$C_{14:0}$	8.13	12.3***	14.6***	6.98***	0.06
$C_{16:0}$	219.20	192.8***	231.8***	197.2***	0.90
$C_{18:0}$	31.00	33.3***	39.4***	25.3***	0.30
$C_{18:1}$	35.00	69.3***	73.9***	42.9	0.51
$C_{18:2}$	178.50	203.3***	183.0**	225.3***	0.90
$C_{18:3}$ n-3	397.50	343.0***	298.0***	408.4***	0.96
SFA (%)	32.40	29.6	34.0	27.9*	1.11
UFA	67.60	70.4	66.0	72.1	1.25
SCFA	1.15	0.79	1.10	0.90	0.25
MCFA	29.50	26.2	29.4	23.7	1.57
LCFA	69.35	73.0	69.5	75.4	1.30

SFA – saturated fatty acids; UFA – unsaturated fatty acids; SCFA – ($C_{6:0}$– $C_{12:0}$); MCFA – $C_{14:0}$– $C_{17:0}$; LCFA > $C_{18:0}$; *E. faecium – Enterococcus faecium*; *L. plantarum – Lactobacillus plantarum*; *L. fermentum – Lactobacillus fermentum*; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ differences from the control

higher ratio is better. The addition of inoculants significantly increased the lactic:acetic ratio from the control silage (9.4) to silages treated with the inoculants CCM 4231 (36.6), LF2 (12.2) and CCM 4000 (13.6; Table 2). A similar effect of either cellulase or cellulase combined with a bacterial inoculant (*Lactobacillus plantarum* and *Pediococcus cerevisiae*) on orchard grass silage was reported by Nadeau et al. (2000). Other results also showed that inoculated grass silages significantly increased (CCM 4231), decreased (CCM 4000) or did not affect (LF2) the ammonia concentration in comparison with control silage. In another experiment (Saarisalo et al., 2007), the other lactic acid bacteria strains (9) as grass silage inoculants showed the ability to limit the production of ammonia-N. It is known that grass is rich in $C_{18:3}$ FA and the ensiling should not decrease its concentration (Doreau and Poncet, 2000). Other authors (Dewhurst and King, 1998) found that the ensiling of forages reduced the PUFA content. The percentage proportions of saturated fatty acids – SFA, unsaturated fatty acids – UFA, as well as short-chain fatty acids – SCFA ($C_{8:0}$ – $C_{12:0}$) and medium-chain fatty acids – MCFA ($C_{14:0}$ – $C_{17:0}$) were similar in all grass silages (Table 3). Only the percentage proportion of long-chain fatty acids – LCFA (> $C_{18:0}$) was numerically (CCM 4231, CCM 4000) higher in comparison with control silage. As expected, conjugated linoleic acid (CLA, *cis* 9, *trans* 11 $C_{18:2}$) and *trans*-vaccenic acid (TVA, *trans* 11 $C_{18:1}$) were not detected in grass silages. They are produced as the intermediary products from rumen biohydrogenation of $C_{18:2}$ and $C_{18:3}$ fatty acids (Jenkins, 1993). Out of the three main fatty acids, the concentrations (mg/g of total FA) of palmitic acid ($C_{16:0}$) in inoculated silages were significantly ($P < 0.001$) lower (CCM 4231, CCM 4000) or higher ($P < 0.001$) in LF2 and, those of linoleic acid ($C_{18:2}$) were significantly ($P < 0.001$) higher in all inoculated silages. The concentration of α-linolenic acid ($C_{18:3}$) was lower ($P < 0.001$) in inoculated silages (CCM 4231; LF2) or higher ($P < 0.001$) in CCM 4000 compared to control silage. The inoculant bacteria were established suf-

Table 4. The counts of inoculants, enterococci and lactic acid bacteria during ensiling and in silage

Silages	Inoculants[a]	Enterococci[b]	LAB[c]
Day 0–1			
Control	Nd	1.10 ± 0.00	1.30 ± 0.00
EF CCM4231	7.00 ± 0.00	1.29 ± 0.19	1.30 ± 0.00
LF2	5.00 ± 0.00	2.10 ± 0.00	2.60 ± 0.00
CCM4000	7.00 ± 0.00	2.10 ± 0.00	1.90 ± 0.00
Day 7			
Control	Nd	6.75 ± 0.07	6.07 ± 0.57
EF CCM4231	7.85 ± 1.30	8.10 ± 0.00***	8.29 ± 0.44***
LF2	5.75 ± 0.23	8.00 ± 0.71***	7.54 ± 0.50***
CCM4000	8.04 ± 0.00	7.02 ± 0.04	8.54 ± 0.50***
Day 21			
Control	Nd	8.05 ± 0.21	8.34 ± 0.21
EF CCM4231	9.40 ± 0.30	9.20 ± 0.10	9.90 ± 0.21**
LF2	8.69 ± 0.39	9.60 ± 1.03	9.35 ± 0.12*
CCM4000	7.55 ± 0.21	10.02 ± 0.55	8.13 ± 0.01
Day 40			
Control	Nd	7.79 ± 0.68	6.50 ± 0.20
EF CCM4231	7.35 ± 0.03	9.60 ± 0.00	8.19 ± 0.29
LF2	6.80 ± 0.10	7.74 ± 0.74	6.37 ± 0.37
CCM4000	7.55 ± 0.55	5.55 ± 0.55	5.95 ± 0.18
Day 105			
Control	Nd	6.40 ± 0.30	8.79 ± 0.34
EF CCM4231	3.10 ± 0.00	4.85 ± 0.62	5.89 ± 0.22
LF2	5.31 ± 0.94	7.89 ± 0.42	7.98 ± 1.38
CCM4000	6.82 ± 0.41	7.54 ± 1.07	7.00 ± 0.53

***$P < 0.001$ (compared to the control); **$P < 0.01$; *$P < 0.05$; Nd – not determined;
[a]inoculated strains selected on M-Enterococcus agar and MRS agar with rifampicin; [b]enterococci; [c] lactic acid bacteria, the counts are expressed in log 10 cfu/g ± SD

ficiently during ensiling (Table 4). Their counts dominated on day 21 of ensiling: CCM 4231 strain amounted to 9.40 ± 0.30 (log10) CFU/g followed by LF2 (8.69 ± 0.39 CFU/g) and by CCM 4000 (7.55 ± 0.39 CFU/g). However, on day 105 (the end of ensiling) the highest counts of *L. plantarum* CCM 4000 were determined (Table 4); but all inoculants were still determined in sufficient amounts in the silage. Marciňáková et al. (2008) reported for silage 9.15 log10 CFU/g of the probiotic strain of *E. faecium* EF9296 (isolate from silage, Marciňáková et al., 2004) used as an inoculant for grass silage. To compare the counts of enterococci and LAB on day 7 with their counts in control silage, the inoculation with CCM 4231 increased their counts significantly ($P < 0.001$). The same situation was found in the silage with LF2 strain as well as with CCM 4000 strain concerning the counts of LAB (Table 4). This ratio was then slightly changed because it was probably influenced by the organic acid concentration in

the competitive relations although the inoculants accounted for a predominant portion of the total enterococci or lactic acid bacteria. As for the pH, it was similar in the inoculated silages as well as in the control silage.

CONCLUSIONS

The microbial inoculants generally had a positive effect on orchard grass silage characteristics in terms of lower pH and higher lactic acid concentration. The inoculants significantly increased the lactic to acetic acid ratio in inoculated silages. The total concentration of acids (acetic, propionic, n-butyric, lactic acid) was 2–3 times higher in inoculated silages compared to control silage. The bacterial inoculants were established in the grass silage very well. Therefore, in subsequent experiments these inoculated grass silages with the inoculants *E. faecium* CCM 4231, *L. fermentum* LF2 and *L. plantarum* CCM 4000 will be used as the components of ruminant ration *in vitro* (artificial rumen) and *in vivo* (in cows) to study their effect on the production of polyunsaturated fatty acids and their isomers – CLA and TVA (*trans*-vaccenic acid) in rumen fluid and in milk of ruminants.

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