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Inoculant effects on red clover silage: fermentation products and nutritive value

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Abstract. The study investigated effects of five different microbial inoculants on silage fermentation and nutritive value. Silage was prepared from red clover-rich material with dry matter content after 24 hours of wilting of 170 g kg^{-1} for the first cut and 430 g kg^{-1} for the second cut. Tests with five different commercial inoculants were based on different strains of Lactobacillus plantarum which were used alone or in combinations with other lactic acid bacteria (1/BO, 2/BI, 3/SI, 4/EC, 5/BM), and chemical additive (CHEM) were used. Six commercial additives were compared with the untreated control. The additives were applied to fresh forage at the levels recommended by the manufacturers.

Chemical compositions of the first and second cut of red clover were significantly different − crude protein 176 g kg^{-1} and 143 g kg^{-1}; NDF 366 g kg^{-1} and 503 g kg^{-1} DM respectively.

In the first trial, silages treated with 2/BI had lower levels of acetic acid 25.5 g kg^{-1} and ethanol 8.0 g kg^{-1} compared to the control values of 35.6 g kg^{-1} and 11.6 g kg^{-1} (P<0.05). Otherwise, the pH, and contents of ammonia nitrogen, ethanol and organic acids were no different from the control silage.

In the second trial, silage treated with 1/BO and 4/EC showed the highest contents of lactic acid. Compared to the untreated control silage, the acetic acid content was lower in silages treated with 2/BI, 3/SI and 5/BM (P<0.05). The lactic acid:acetic acid ratio was higher in inoculated silages: for 1/BO, 2/BI; 3/SI, 4/EC and 5/BM it was 2.73; 2.17; 1.98; 2.03 and 2.87, respectively. The same ratio for the control silage was 1.83. All commercial inoculants improved the fermentation quality of red clover silage under the conditions stated.

No differences were found between dry matter *in vitro* digestibility of the inoculated and the control silage for both the first and second cut. Digestibility of the red clover silage treated with CHEM was higher than that of the control silage by 4.6% for the first cut and by 7.3% for the second cut (P<0.001).

Key words: inoculants, red clover, silage, fermentation, nutritive value, digestibility

INTRODUCTION

Silage is an increasingly important forage for dairy cows. It is also the most variable and unstable feedstuff used in dairy cattle diets. In Estonia as well in the other Baltic and Scandinavian states, red clover and red clover-timothy mixture – with a high nutritive value but with low ensilability – are commonly grown for silage (Rinne & Nykänen, 2000, Hetta et al., 2003, Jatkauskas & Vrotniakiene, 2006). In order to improve the nutritive value and hygienic quality of forages, several silage additives have been developed (Muck & Kung, 1997). Biological additives consisting essentially of different strains of lactic acid bacteria have been widely used to initiate silage fermentation. Homofermentative strains such as *Lactobacillus plantarum, Enterococcus faecium* and *Pediococcus spp*. produce the largest reductions in pH, higher lactate:acetate ratios, lower ethanol and ammonia-nitrogen contents, and lower dry matter losses by 1-2% (Weiberg & Muck, 1996). Heterofermentative strains of lactic acid bacteria such as *Lactobacillus buchneri* produce high levels of acetic acid that inhibit fungi and improve aerobic stability (Filya, 2003; Kung et al., 2003a). Despite the difference in inoculants, they all promote silage fermentation and preserve the nutritive value of the grass feed. The chemical composition and digestibility are important factors in predicting the voluntary intake of silage. As feed intake is one of the major limiting factors in cattle nutrition, its increase can improve animal productivity (O'Kiely, 1996, Huhtanen et al., 2002, Jatkauskas et al., 2002, Rondahl et al., 2007).

Several different biological additives can be used for making silage. The described research was aimed at finding silage additives suitable for treating red clover-rich grass cultivated under Estonian climatic conditions. Their effects on silage fermentation characteristics, nutritive value and digestibility were also investigated.

MATERIAL AND METHODS

The red clover-grass mixture (75% red clover *Trifolium pretence* L. *subvar*. *praecox* Witte variety 'Jõgeva 433' and 25% timothy *Phleum pratence* L. variety 'Tika') at the stage of bud formation at the first cut and at the stage of ear formation at the second cut from the field of Tartu Agro was used. Grass was ensiled after wilting in 2 trials on 2007 June 7 and July 30, respectively. The material, cut with mowing machine and after wilting one day on the field, was chopped into 2 cm lengths with laboratory machinery, mixed and conserved in 3−litre glass jars. There were 3 replicates. The first trial comprised 6 treatments (untreated control, 4 inoculants and chemical additive); the second trial, 7 treatments (untreated control, 5 inoculants and chemical additive). The additives were commercial products (inoculants 1 to 5; Table 1). The additives were applied to fresh forage at the levels 1 g/tonne of 1/BO, 2/BI, 5/BM and 10 g/tonne 3/SI and 5,25 g/tonne 4/EC and 5 litres/tonne CHEM.

Table 1. Inoculants used in the trials

Number Code	Inoculant	Source
1/BO	*L. plantarum*, *P. pentosaceus, L. rhamnosus, L. brevis, L. buchneri*	Schaumann Agri Austria GmbH & Co KG
2/BI	*L. plantarum*	Dr. Pieper Ltd. Wuthenow, Germany
3/SI	*L. plantarum, E. faecium, P. acidilactici, L. salivarius*	Alltech Biotechnoligy Centre, Co. Meath, Ireland
4/EC	*L. plantarum* MTD1	Ecosyl, Yorkshire, UK
5/BM	*L. plantarum* and *P. pentosaceus*	Chr. Hansen Biosystems, Denmark

The jars were opened for analysis after 90 days.

In order to determine the content of volatile fatty acids, ethanol and ammonia nitrogen, and the pH value, a solution of each silage sample was prepared: 50 g silage was weighed, 100 ml distilled water added and it was filtered through a paper filter.

The pH value was measured with a Hanna Instruments Mikroprocessor pH meter 210; ammonia nitrogen was determined using an adjusted Kjeltec 2300 (FOSS) analyser. The ethanol, lactic acid and volatile fatty acid contents were determined chromatographically using an Agilent Technologies 7890A GC system with a column packed with 80/120 Carbopack B-DA/4% carbowax 20 M (Faithfull, 2002).

Samples were dried for 20 hours at 60°C, chopped into 1 mm-diameter particles and analysed for the DM, crude protein, crude ash and crude fibre (AOAC, 2005). For determining crude ash concentration, samples were reduced to ashes in a furnace at 550°C for 6 hours. Crude protein was analysed by the Kjeldahl method with Kjeltec 2300 analyser (FOSS Tecator Technology). Dried-ground (1 mm) samples were analysed for *in vitro* digestibility. *In vitro* digestibility of DM (IVDMD) was determined after incubating forage samples in buffered rumen fluid for 48 h using a DAISY II Incubators (ANKOM Technology, Fairport, NY USA). The buffer was prepared according to the ANKOM Technology procedure. The rumen fluid was obtained before feeding from 2 fistulated dairy cows, fed red clover-grass silage *ad libitum* and barley-cut meal 9 kg. The NDF and ADF concentrations of the samples and digested residues were determined with amylase pre-treatment using an ANKOM 220 Fiber Analyzer (ANKOM Technology) (Van Soest *et al*., 1991).

Water soluble carbohydrates (WSC) were determined by the Thomas anthrone method (1977) and buffering capacity (BC) by titration of lactic acid to pH 4.0.

Because of differences in initial quality characteristics between harvests, statistical analysis was performed for each cut separately with the generalized linear model procedure of SAS. The effects of treatments were tested by means of orthogonal contrasts. Analysing the traits containing zero values, ranks of values were used; other traits were transformed to their logarithmic values.

RESULTS AND DISCUSSION

Chemical compositions of the first and second cut of red clover were significantly different. The first cut red clover-rich grass at the stage of bud formation was difficult to wilt because it was rainy and its dry matter remained low. The second cut material was harvested later and the DM was higher. In the first cut red clover the crude protein, crude ash and water soluble carbohydrates contents were higher, but the NDF and ADF contents were lower than those of the second cut (Table 2). Chemical composition was significantly affected by climatic conditions during the growing period and the time of harvesting.

Table 2. Chemical composition (g kg^{-1} of DM) and buffering capacity of the red clover-grass material before ensiling

Cu	DM	Crude protein	Crude ash	NDF	ADF	N−free extractives	Water soluble carbohydrates	Bufferi capacit
I	170	176	102	366	260	468	68	78
II	430	143	98	503	330	451	50	56

Fermentation characteristics, pH, ammonia nitrogen in total nitrogen, organic acids and ethanol contents are given in Tables 3 and 4. No butyric acid was found in the test silages made from the first cut material. In the first trial, pH and ammonia nitrogen, ethanol and organic acid contents were no different from those of the control silage except for the CHEM-treated silage. As is shown in Table 3, silages treated with 2/BI were exceptions as they had lower levels of acetic acid and ethanol (P<0.05).

Biosil does not contain the *L. buchneri* strain which produces high concentrations of acetic acid in silages (Filya et al., 2007). In both trials, the characteristics of silages treated with CHEM differed from those of silages inoculated with biological additives as well as from the uninoculated control silage by the lower content of organic acids (<0,05) and ethanol (<0,05), but higher ammonia nitrogen (<0,01) concentration. AIV Pro contained ammoniumformiate (30,3%). This explains the high ammonia nitrogen concentration in the CHEM silages. This was predictable as chemical additives have an inhibiting effect on fermentation (Wilkinson, 2005).

Table 3. Fermentation characteristics of the first cut red clover-grass silages in dry matter

Treatment	Dry matter, g kg^{-1}	pH	Ammonia-N, % of total N	Lactic acid, g kg^{-1}	Acetic acid, g kg^{-1}	Butyric acid g kg^{-1}	Ethanol, g kg^{-1}
Control	157	4.17	3.77	66.9	35.6	0,0	11.6
1/BO	159	4.23	4.03	74.0	37.6	0,0	11.3
2/BI	163	4.10	3.00	62.6	25.5	0,0	8.0
3/SI	151	4.20	3.17	65.4	35.6	0,0	10.8
4/EC	146	4.30	3.97	44.7	35.5	0,0	9.6
CHEM	150	4.27	7.93	30.0	13.2	0,0	0.7
Significant difference, *P*							
C vs 1/BO	0.016	0.115	0.329	0.355	0.197	-	0.344
C vs 2/BI	0.006	0.058	0.051	0.289	0.004	-	0.001
C vs 3/SI	<0.001	0.187	0.081	0.439	0.494	-	0.151
C vs 4/EC	<0.001	0.008	0.308	0.028	0.477	-	0.065
C vs CHEM	<0.001	0.051	0.002	0.023	<0.001	-	<0.001

Table 4 shows that in the second trial, ammonia nitrogen and pH values of the silages with biological inoculants were lower (4.10−4.30) than those of the control silage (4.37).

Compared to the control silage, the lactic acid content was higher in silages treated with 1/BO and 4/EC, whereas the content of acetic acid was lower in silages inoculated with 2/BI, 3/SI and 5/BM (P<0.05). The lactic acid:acetic acid ratio in inoculated silages was higher than that in the control silage (1.83) – for 1/BO, 2/BI, 3/SI, 4/EC and 5/BM: 2.73; 2.17; 1.98; 2.87 and 2.03, respectively. Most inoculant LAB treatments produced silages with higher lactate:acetate ratios than the uninoculated control. Similar changes in silage fermentation, which accompany the use of homofermentative LAB strains, have previously been shown by several authors (Weinberg & Muck, 1996; Kung et al., 2003). The changes have not always been observed for the strains of *L. buchneri* ja *E. faeciumi* (Filya et al., 2007).

Table 4. Fermentation characteristics of the second cut red clover silages in dry matter

Treatment	Dry matter g kg^{-1}	pH	Ammonia-N, % of total N	Lactic acid g kg^{-1}	Acetic acid g kg^{-1}	Butyric acid g kg^{-1}	Ethanol g kg^{-1}
Control	420	4.37	4.63	48.8	26.7	0.5	5.5
1/BO	413	4.10	4.03	68.9	25.2	0.2	4.7
2/BI	397	4.17	4.13	48.7	22.4	0.2	4.7
3/SI	412	4.30	4.43	46.2	23.3	0.0	4.2
4/EC	386	4.23	3.80	73.2	25.5	0.3	4.2
5/BM	392	4.30	4.53	50.6	24.9	0.0	4.8
CHEM	411	4.40	6.33	17.3	16.3	0.0	2.8
Significant difference *P*							
C vs 1/BO	0.012	<0.001	<0.001	0.022	0.062	<0.001	0.033
C vs 2/BI	0.006	0.007	0.078	0.494	0.011	<0.001	0.187
C vs 3/SI	0.017	0.058	0.193	0.390	0.006	<0.001	0.070
C vs 4/EC	<0.001	0.024	0.003	0.036	0.267	<0.001	0.003
C vs 5/BM	<0.001	0.058	0.346	0.382	0.042	<0.001	0.049
C vs CHEM	0.039	0.322	<0.001	0.001	<0.001	<0.001	0.023

Table 4 shows that in the test silages of the second cut the butyric acid content was lower than that of the untreated control silage (P<0.001). The positive effect of inoculants on the fermentation process of red clover-rich grass has been claimed by several researchers (Winters et al., 2002, Gallo et al., 2003, 2006; Kaldmäe et al., 2007).

On average, crude protein, NDF and ADF concentrations of silages in both cuts (Tables 5 and 6) were higher and N-free extracts lower than their respective values before ensiling (Table 2). Dry matter losses and the use of sugars by microorganisms during fermentation change nutrient concentrations (Pahlow et al., 2003).

Table 5. Chemical composition (in DM) and *in vitro* digestibility (IVDMD) of first cut red clover rich silages.

Treatment	Crude protein g kg^{-1}	NDF g kg^{-1}	ADF g kg^{-1}	N−free extractives g kg^{-1}	IVDMD %
Control	183	417	303	453	76.2
1/BO	188	418	305	450	74.8
2/BI	183	412	307	456	73.5
3/SI	184	419	310	458	74.0
4/EC	184	413	312	451	75.8
CHEM	189	411	300	445	80.8
Significant difference, *P*					
C vs 1/BO	0.037	0.473	0.222	0.187	0.177
C vs 2/BI	0.449	0.160	0.048	0.241	0.063
C vs 3/SI	0.432	0.389	0.002	0.046	0.239
C vs 4/EC	0.447	0.198	0.083	0.273	0.410
C vs CHEM	0.059	0.176	0.281	0.013	0.055

Dry matter digestibility of inoculated silages did not show any difference between the control (P>0,05) (Tables 5 and 6). The silage of the first cut had higher DM digestibility than the second cut, but the variations are not significant. The material was cut differently, and at a different stage of vegetation. It is well documented that increases in DM yield of growth are accompanied by a decrease in herbage digestibility and nutrient concentrations (Rinne & Nykänen, 2000). However, silages treated with CHEM showed a slight increase in digestibility. Digestibility of the red clover-rich silage treated with CHEM was significantly higher than that of the control silage – by 4.6% for the first cut and by 7.3% for the second cut (<0,001). The positive effect of inoculants on silage fermentation but not on digestibility has been reported by Weinberg & Muck (1996). Inoculation had no effect on digestibility of high quality silages (-.07) but improved digestion of lower quality forage by 1.57 units. Effects of inoculation on digestibility were less than the effects of formic acid for all comparisons (Harrison et al., 1994, Jatkauskas & Vrotniakiene 2006). Filya et al (2007) report that only 30% of the trials have shown increased digestibility.

Table 6. Chemical composition (in DM) and *in vitro* digestibility (IVDMD) of second cut red clover-rich silages

Treatment	Crude protein, g kg^{-1}	NDF, g kg^{-1}	ADF, g kg^{-1}	N−free Extractives, g kg^{-1}	IVDMD, %
Control	149	532	354	437	63.4
1/BO	144	547	353	440	62.1
2/BI	143	545	354	442	64.9
3/SI	148	539	352	428	64.4
4/EC	151	522	374	441	62.7
5/BM	145	527	370	441	64.2
AIV Pro	150	528	361	438	70.7
Significant difference *P*					
C vs 1/BO	0.012	0.002	0.361	0.294	0.227
C vs 2/BI	0.008	0.025	0.493	0.216	0.115
C vs 3/SI	0.374	0.105	0.176	0.052	0.246
C vs 4/EC	0.096	0.002	0.028	0.222	0.347
C vs 5/BM	0.181	0.162	0.002	0.260	0.238
C vs CHEM	0.282	0.157	0.014	0.492	<0.001

CONCLUSIONS

This study shows that the use of additives for ensiling red clover-rich pre-wilted material improves the dry matter concentration of silage (decrease DM losses). The use of a chemical additive for ensiling red clover-rich grass, especially with the low dry matter concentration, improved silage quality: butyric acid, ethanol showed a decrease, whereas the lactate**:**acetate ratios were increased. Dry matter digestibility of the inoculated silages did not increase while the chemically treated silage improved in both trials.

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