The effect of a mixture of Lactobacillus strains on silage quality and nutritive value of grass harvested at four growth stages and ensiled for two periods

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The effect of adding an inoculant containing *Lactobacillus buchneri, L. plantarum and L. casei* to wilted perennial ryegrass, harvested at four growth stages and ensiled for either 60 or 150 d on silage fermentation quality, chemical composition, rumen degradability of neutral detergent fibre (NDF) and organic matter (OM) and in vitro OM digestibility (OMd) was studied. Compared to the control silage, more sugars were fermented to lactic and acetic acid with the inoculant, resulting in a lower pH, less dry matter losses and protein degradation and a better aerobic stability. The effects of the additive on fermentation quality were more pronounced after 150 than after 60 d of ensiling, because the quality of the control silage was worse after the long ensiling period. The treatment lowered NDF content of grass harvested at the first two growth stages by degrading cell walls to complex sugars, but had no effect on NDF degradability of the silage. The inoculant had no effect on rumen OM degradability nor on OMd after the short ensiling period, but increased the rumen OM degradability for the first two growth stages and OMd for all growth stages after the long ensiling period.

Key words: cell wall degradability, ferulate esterase, *Lactobacillus,* grass silage

Introduction

Grass silage is an important component in the ration of cattle especially during the winter time. The quality of grass silage depends to a large extent on the growth stage of the grass at harvest, the weather conditions during field wilting and the ensiling practices. In case of unfavourable ensiling conditions, silage additives may be used. Currently mostly inoculants containing living micro-organisms are used. Recently an inoculant for ensiling grass was introduced with the aim to improve not only silage fermentation quality and aerobic stability, but also to enhance digestibility and nutritive value of the silage. This so-called multipurpose inoculant consists of three Lactobacillus strains: *L. plantarum, L. casei* and *L. buchneri*. The former two strains are facultative heterofermentative, whereas the latter is strict heterofermentative (Holzer et al. 2003). The production of acetic acid besides lactic acid explains the improvement of aerobic stability, observed with the use of *L. buchneri* (Kung and Ranjit 2001). Moreover, *L. buchneri* is able to produce ferulate esterase (FE), an enzyme which breaks down the linkages between (hemi)cellulose and lignin (Donaghy et al. 1998).

The objective of this study was to examine if the above-mentioned aims are reached by investigating the effects of the inoculant on the fermentation characteristics, the chemical composition, the in situ rumen degradability and in vitro digestibility of grass silage. Although it is recommended to mow grass not too late for making good quality silage, the efficacy of the inoculant was studied at four growth stages. It would answer the question if the claimed positive effect of the additive on digestibility offers farmers more flexibility to postpone harvest in case of bad weather conditions or to obtain higher grass yields. The effect of inoculant additive was studied after 60 and 150 days of ensiling to examine if a long ensiling period could have an additional effect compared to a more common ensiling period. This additional effect is expected as *L. buchneri* is known as a slowly growing bacterium (Schmidt et al. 2009).

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Material and methods

The grass used in the experiment originated from a parcel perennial ryegrass (*Lolium perenne*) sown in April 2008, which received 120 kg N ha^{-1} in March 2010. The first cut of the grass was weekly harvested between the end of April and early June 2010. Then, 4 stages were selected for further study based on distinct contents of NDF. At each harvest a representative batch of about 150 kg grass was mown with a Haldrup harvester (Inotec, Løgstør, Denmark) around 1100 h. Dry matter (DM) yield was estimated from the fresh weight of the harvested area and the DM-content of the grass. Then, grass was wilted on the field to obtain a DM-content of about 35%. Wilting lasted for 25, 29, 73 and 24 h, respectively; the longer wilting period after the third harvest was because of rain on the second day. Subsequently, the grass was cut with a field chopper (Sperry New Holland, Zedelgem, Belgium) set at a theoretical length of 24 mm. Samples of the wilted grass were taken, dried in a ventilated oven at 65 °C, ground to pass a 1-mm screen and analysed for residual moisture, neutral detergent fibre (NDF) and sugar content. NDF was determined with the filter bag method using α-amylase and sodium sulphite (Van Soest et al. 1991). Sugars were extracted with 40% ethanol (EC 1971a).

The wilted grass was ensiled in cylindrical plastic tubes with a volume of 2.75 l (height 35 cm, diameter 10 cm) and provided with a CO_2-lock at a density of about 180 kg DM per m^3. Ten micro-silos were made for both the control and the treatment. For making the treated silos, 20 kg wilted grass spread on a plastic sheet was sprayed with 0.2 l of distilled water in which 20 mg of inoculant additive was dissolved (recommended dose: 1 g per ton wilted grass) and thoroughly mixed. The studied inoculant Pioneer ® 11GFT is a commercial product (Pioneer Hi-bred Northern Europe) containing the strains *L. buchneri* LN40177, *L. plantarum* LP24011 and *L. casei* LC32909 at concentrations of >1.0 x 10^{11}, >2.0 x 10^{10} and >1.0 x 10^{10} cfu per g product, respectively. Before filling the control silos, 0.2 l distilled water was added to another 20 kg of wilted grass. The number of lactic acid bacteria (LAB) in the inoculant as well as in the control and inoculated silage material was counted according to ISO 15214 (1998). The filled micro-silos were stored at ambient temperature in an enclosed barn. Half of the tubes were ensiled for 60 d, the other half for 150 d.

Micro-silos were weighed weekly to determine fermentation losses. Eighteen days before opening, aerobic stress was induced to all micro-silos by removing the tape from openings at the bottom and top of the tubes during 24 h. At the opening of the silos, 4 out of 5 tubes were selected for further study by eliminating the micro-silo with visible most mould growth. The remaining tubes were individually sampled to determine silage quality. Aerobic stability was measured according to Honig (1990) for a maximum of 170 h and expressed as the time in hours until temperature of silage raised 3 °C above ambient temperature. To determine fermentation characteristics an extract was made by soaking 100 g of silage in 1 l distilled water at 4 °C during 16 h. On the extract pH, ammonia-N (KjeldahI without previous destruction, ISO 5983-2 2005), lactic acid (Noll 1966, Gawehn 1984), acetic acid, propionic acid, butyric acid and alcohols with gas chromatography (Jouany 1981) were determined.

The effect of additive on rumen degradability of NDF and OM was studied on 3 out of 4 randomly selected micro-silos per control/treatment. Also, DM, crude ash and NDF were analysed on each micro-silo. The DM-content was determined by drying a sample in a ventilated oven at 65 °C and analysis of residual moisture in a ground subsample by drying at 103 °C during 4 h (EC 1971b). Crude ash content was obtained by incineration at 550 °C (ISO 5984, 2002). The rumen degradation characteristics of OM and NDF were determined by means of the nylon bag technique. Therefore, bags (8 x 10 cm, pore size 37 µm) were filled with 2.5/5.0 g DM-equivalent of frozen and finely cut silage (particles ≤ 10 mm) and incubated in two rumen-cannulated cows for 8, 24, 48, 72 and 336 h (a two-fold sample weight was used for the two long incubation times). Per time four bags, two per cow, were incubated. Besides, 3 bags were filled with sample but underwent no rumen incubation to determine the wash-out fraction (W). The lactating cows received a basal ration consisting of maize and grass silage (50/50 on DM-basis) supplemented with concentrates. After incubation, bags with residues were rinsed under running tap water, frozen, machine-washed (Zanussi, Frankfurt/Main, Germany) with cold water for 50 min without spin cycle and freeze dried. Residues were pooled per incubation time and ground to pass a 1-mm screen. Incubation residues were analyzed for residual moisture, crude ash and NDF. The potentially degradable fraction (D) was calculated as 100 - W - U, with U being the undegradable fraction after 336 h of incubation.

The degradation rate (kd) of D was derived by iteration using the exponential model $d(t) = W + D \times (1 - e^{(-kdxt)})$ with d(t) the disappearance at time t (Ørskov and McDonald 1979). Then, the rumen fermentable NDF fraction (FNDF) was calculated as:

$$FNDF\ (\%) = D_{NDF} \times [kd_{NDF}/(kd_{NDF} + kp_{NDF})]$$

with kp_{NDF} being the passage rate of NDF derived from the equation: $kp_{NDF} = 0.1775 \times kd_{NDF} + 1.39$ and assuming $W_{NDF} = 0$ (Tamminga et al. 2007).

The rumen fermentable OM (FOM) fraction was calculated as:

$$FOM\ (\%) = W_{OM} + D_{OM} \times [(kd_{OM}/(kd_{OM} + kp_{OM})]$$

with kp_{OM} the passage rate of OM, equaling 4.5 % h^{-1} (Tamminga et al. 2007).

Finally, the remaining material of the 3 micro-silos was pooled per treatment, dried and ground to pass a 1-mm screen. Crude protein (CP; ISO 5983-2, 2005), sugars and crude fat (ISO 6492, 1999) were determined and total OM digestibility (OMd) was estimated by an in vitro cellulase technique (De Boever et al. 1986). The content of residual non starch polysaccharides (RNSP), as a measure of pectins and complex sugars like arabans, xylans and beta-glucans (Tamminga et al. 2007) was calculated as:

RNSP = 1000 - NDF - CP - crude ash - sugars - crude fat - FP

with FP being the silage fermentation products calculated as the sum of lactic, acetic, propionic and butyric acid as well as the alcohols.

The DM-content and chemical composition were corrected for losses of volatile substances according to Dulphy and Demarquilly (1981).

Statistical analysis was carried out by means of SAS for Windows version 9.3. The normality for the repetitions within control/treatment was examined according to Kolmogorov-Smirnov. If normal, an ANOVA was done to investigate the effect of the additive treatment and the interaction between additive treatment and growth stage; control and treatment means within growth stage and ensiling period were compared by a t-test. If not normal, a non-parametric Kruskall-Wallis analysis was carried out to investigate the effect of treatment and to compare control and treatment means; this was only the case for pH after 60 and 150 d and for butyric acid content after 150 d. For the parameters determined on a pooled silage sample, CP, sugars, crude fat and OMD as well as the sum of the fermentation products and RNSP, the overall effect of treatment was examined across the 4 growth stages within ensiling period using ANOVA.

Results

From the first to the last harvest date DM-yield increased from 3100 to 5100 kg and NDF content from 365 to 535 g kg^{-1} DM (Table 1). Sugar content varied in the range of 150 g kg^{-1} DM at the third growth stage to 225 g kg^{-1} DM at the first growth stage.

Table 1. Dry matter yield and chemical composition of grass harvested at 4 growth stages

Harvest date	Yield (kg DM[a] ha^{-1})	DM (g kg^{-1})	NDF[b] (g kg^{-1} DM)	Sugars (g kg^{-1} DM)
28 April 2010	3100	364	365	225
17 May 2010	3450	336	422	184
25 May 2010	4010	365	505	150
2 June 2010	5100	360	535	201

[a] dry matter

[b] neutral detergent fibre

The number of LAB in the applied product amounted to 1.3 $x10^{10}$ cfu g^{-1}, which was somewhat lower than the number mentioned on the label (1.1 x 10^{11} cfu g^{-1}). Following the recommendation of the manufacturer 1.3 x 10^4 cfu was effectively added per kg of wilted grass. Compared to the control silage addition of the inoculant increased the number (cfu g^{-1} wilted grass) of lactic acid bacteria (ISO 15214) from 6.0 x 10^2 to 3.2 x 10^4 at the first stage, from 4.8 x 10^2 to 3.0 $x10^4$ at the second, from 2.0 x 10^3 to 1.5 x 10^4 at the third and from <10 to 1.2 x 10^4 at the last growth stage.

Chemical composition and in vitro OM digestibility

The DM-content of the control silage either after 60 d or 150 d of ensiling (Table 2) corresponded fairly well with that of the wilted grass at ensiling (Table 1). The addition of the inoculant significantly ($p \leq 0.01$) increased DM-content at all stages and for both ensiling periods. The increase was most pronounced for stages 1 and 2 after both 60 and 150 d of ensiling.

Compared with the grass at ensiling, the NDF content of the control silages after both 60 and 150 d of ensiling was somewhat lower. Treatment with the inoculant significantly ($p \leq 0.01$) decreased NDF content at the first two growth stages after 60 d of ensiling and the first three stages after the long ensiling period.

The ash content of the control silage was significantly ($p \leq 0.05$) higher than that of the treated silages. The difference was smallest at the last growth stage.

The protein content of the control silage decreased with later harvesting from 238 g kg^{-1} DM for stage 1 to 132 g kg^{-1} DM for stage 4. Treated silages had a lower CP content than the control silages; averaged over the four growth stages the difference amounted to 4 and 10 g kg^{-1} DM for periods of 60 and 150 d of ensiling, respectively, but significantly ($p \leq 0.05$) only after 60 d.

The sugar content of the control silage after 60 d was clearly lower compared with that of the grass at ensiling except for stage 2; longer ensiling further decreased sugar content. All treated silages had a lower sugar content than the control silage. Averaged over the four growth stages the sugar content of the control and treated silages after the 60 d of ensiling amounted to 95 and 35 g kg^{-1} DM, respectively, and after the 150 d of ensiling to 45 and 23 kg^{-1} DM, respectively. The difference was significant ($p \leq 0.05$) only for the short ensiling period.

The content of fermentation products (FP) of the control silage after 60 d varied between 78 g kg^{-1} DM at stage 2 to 98 g kg^{-1} DM at stage 4. Longer ensiling further increased FP for stages 1 and 2. Compared with the control silage, the inoculant significantly increased FP content from 91 to 125 g kg^{-1} DM after 60 d and from 103 to 131 g kg^{-1} DM after 150 d.

Crude fat content of all silages varied between 38 and 49 g kg^{-1} DM and was not affected by the treatment.

The content of RNSP tended to increase with later harvesting and longer ensiling. The inoculant increased the RNSP content in all cases, except for the fourth growth stage after 150 d of ensiling. Averaged over the four growth stages the RNSP content of the control and treated silage after the 60 d of ensiling amounted to 77 and 124 g kg^{-1} DM, respectively, and after the 150 d of ensiling to 102 and 124 kg^{-1} DM, respectively. The difference was significant ($p \leq 0.05$) only after the short ensiling period.

The cellulase OM digestibility of the control silage (averaged for the two ensiling periods) decreased gradually from 91.3% at growth stage 1 to 73.4% at growth stage 4. The OMd was always higher with the inoculant than with the control, except at growth stage 2 after 60 d of ensiling. The mean OMd for the four growth stages of the control and the treated silage after 60 d of ensiling amounted to 82.6 and 83.6%, respectively, and after 150 d of ensiling to 81.6 and 83.9%, respectively. The difference was significant ($p \leq 0.05$) only after the long ensiling period.

Table 2. The effect of the inoculant (control C versus treatment T) on the chemical composition and in vitro OMd of grass harvested at 4 growth stages and after an ensiling period (EP) of either 60 or 150 d (means of 3 micro-silos for DM, NDF and crude ash; single values for the other parameters).

	EP	Stage 1		Stage 2		Stage 3		Stage 4		SEM[e]	T[f]	TxS[g]
	(d)	C	T	C	T	C	T	C	T			
Dry matter	60	387	397**	342	362**	370	380**	359	365**	2.9	**	**
(g kg^{-1})	150	378	404**	334	356**	367	375**	352	358*	3.5	**	**
NDF[a]	60	344	317**	397	377**	491	484^{ns}	513	506^{ns}	15.3	**	**
(g kg^{-1}DM)	150	344	313**	410	379**	492	483*	504	514^{ns}	15.3	**	**
Crude ash	60	111	108*	94	86**	81	75**	81	78^{ns}	2.7	**	**
(g kg^{-1}DM)	150	117	110**	97	87**	82	78**	80	79*	2.9	**	**
Crude protein	60	231	226	169	167	141	138	135	129	13.5	*	nd
(g kg^{-1}DM)	150	245	225	186	167	144	142	129	129	14.7	ns	nd
Sugars	60	119	70	153	38	58	17	52	14	16.1	*	nd
(g kg^{-1}DM)	150	48	47	65	26	36	10	32	9	6.3	ns	nd
FP[b]	60	96	132	78	136	91	120	98	112	6.8	*	nd
(g kg^{-1}DM)	150	118	141	108	143	93	120	94	120	6.3	**	nd
Crude fat	60	41	46	41	46	39	38	41	41	1.0	ns	nd
(g kg^{-1}DM)	150	45	49	46	47	40	43	41	44	1.0	ns	nd
RNSP[c]	60	57	101	70	149	100	127	82	120	10.2	*	nd
(g kg^{-1}DM)	150	83	115	88	150	115	124	120	105	7.0	ns	nd
OMd[d]	60	91.8	92.4	88.8	88.1	76.9	79.1	73.1	74.6	2.64	ns	nd
(%)	150	90.7	93.0	86.8	88.6	75.2	79.0	73.6	74.9	2.61	*	nd

[a] neutral detergent fibre
[b] fermentation products
[c] residual non starch polysaccharides
[d] in vitro cellulase digestibility of the OM
[e]: standard error of the mean
[f]: significance of treatment effect
[g]: significance of interaction between treatment (T) and growth stage (S)
ns: not significant ($p > 0.05$), * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$
nd: the interaction treatment x growth stage could not be determined because of limited degrees of freedom

Silage fermentation quality

The weekly DM-losses of all micro-silos increased linearly ($p \leq 0.01$) from week 1 to the opening of the silos after both 60 and 150 d of ensiling. For the control silages of 60 d ensiling losses increased gradually up to 6 weeks after ensiling but with a steeper slope after the induction of aerobic stress (Fig. 1). The losses were higher with advancing harvest stage. Initially, treated silages had higher DM-losses than control silages, the difference being significant ($p \leq 0.05$) up to weeks 4, 4, 3 and 2 after ensiling for stages 1, 2, 3 and 4, respectively. With longer ensiling, DM-losses for treated silages were no longer different ($p > 0.05$) from control silages and became even smaller ($p \leq 0.05$) after 8, 7 and 4 weeks for stages 1, 3 and 4, respectively.

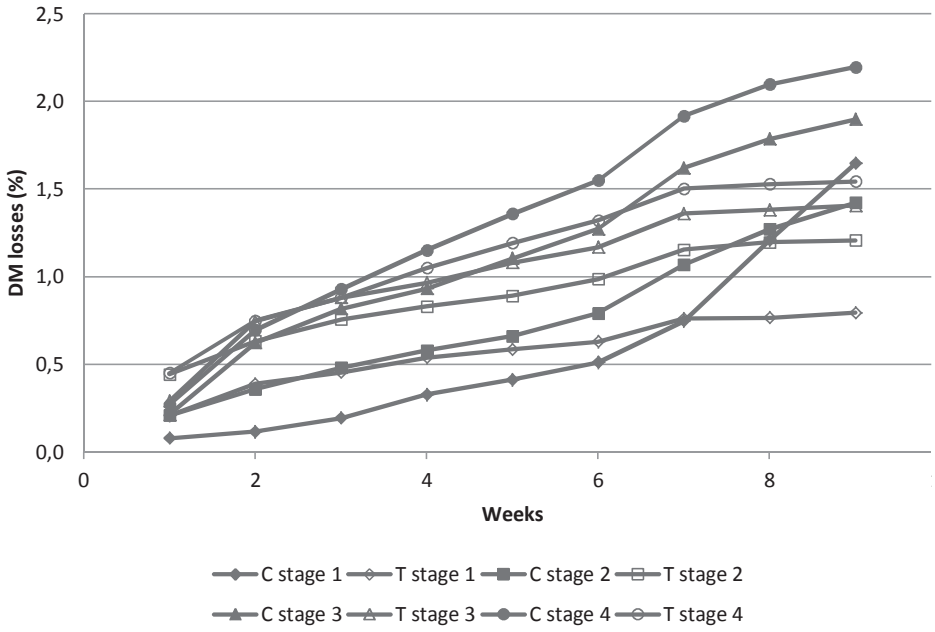

Fig. 1. Weekly DM-losses of control (C) and treated (T) grass silage at four growth stages during a 60 d ensiling period.

The DM-losses of the control silages ensiled for 150 d (Fig. 2) increased gradually for stages 3 and 4, whereas losses for stages 1 and 2 showed a steep increase after about 8 weeks and were higher than those of the later stages at the opening of the silos. Initially, treated silages had higher DM-losses than control silages, the difference being significant ($p \leq 0.01$) up to weeks 3, 3, 2 and 1 after ensiling for stages 1, 2, 3 and 4, respectively. With longer ensiling, DM-losses for treated silages were no longer different ($p > 0.05$) from control silages and became even smaller ($p \leq 0.01$) after 12, 12, 7 and 6 weeks for stages 1, 2, 3 and 4, respectively.

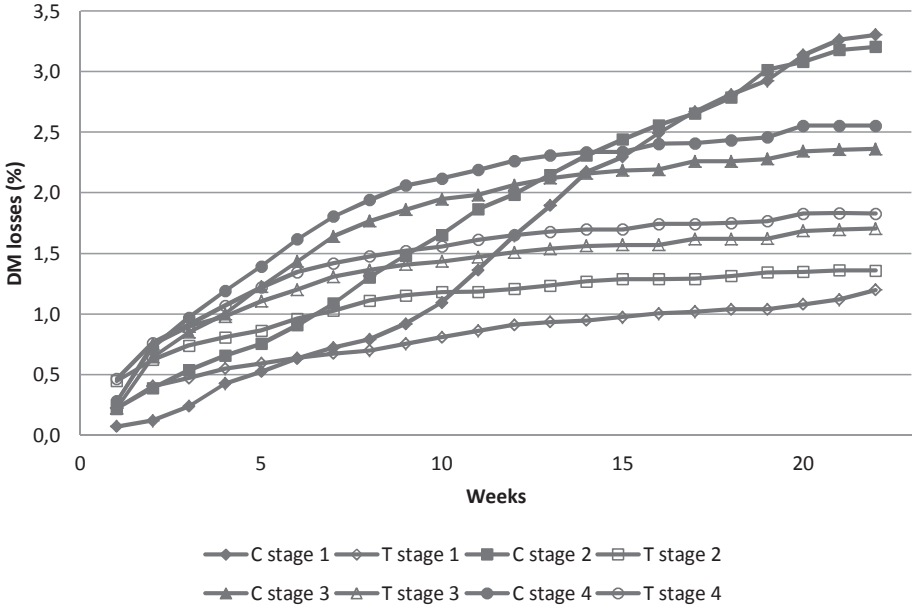

Fig. 2. Weekly DM-losses of control (C) and treated (T) grass silage at four growth stages during a 150 d ensiling period.

The final weight loss of the control silages after 60 d of ensiling varied from 1.4% for growth stage 2 to 2.2% for growth stage 4 (Table 3). Longer ensiling increased weight losses particularly for growth stages 1 and 2, when losses more than doubled. The inoculant significantly ($p \leq 0.01$) decreased losses, the effect being significant for stages 1, 3 and 4 after 60 d of ensiling and for all stages after the long ensiling period.

Table 3. The effect of the inoculant (control C versus treatment T) on silage quality of grass harvested at 4 growth stages and after an ensiling period (EP) of either 60 or 150 d (means of 4 micro-silos).

	EP	Stage 1		Stage 2		Stage 3		Stage 4		SEM[a]	T[b]	TxS[c]
	(d)	C	T	C	T	C	T	C	T			
Final weight	60	1.6	0.8**	1.4	1.2^{ns}	1.9	1.4**	2.2	1.5**	0.08	**	**
loss (%)	150	3.3	1.2**	3.2	1.4**	2.4	1.7**	2.6	1.8**	0.14	**	**
pH	60	4.93	3.93**	4.60	3.84**	4.41	3.93**	4.42	4.04**	0.066	**	nd
	150	5.92	3.95**	5.07	3.85**	4.62	4.01**	4.82	4.07**	0.121	**	nd
Lactic acid	60	32	87**	40	83**	46	71**	45	52^{ns}	3.5	**	**
(g kg^{-1} DM)	150	29	89*	26	87**	34	65**	33	55**	4.5	**	**
Acetic acid	60	26	24^{ns}	11	32**	11	27**	11	34**	1.6	**	**
(g kg^{-1} DM)	150	16	29**	8	33**	16	29**	10	40**	2.0	**	**
Butyric acid	60	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	ns	ns
(g kg^{-1} DM)	150	0.0	0.0^{ns}	0.0	0.0^{ns}	2.3	0.0*	3.3	0.1**	0.26	**	nd
Alcohols	60	38	21**	27	21*	34	22**	42	26**	1.4	**	**
(g kg^{-1} DM)	150	73	24**	74	24**	41	26**	48	25**	3.7	**	**
NH_3-N/N	60	4.5	2.7**	6.3	3.8**	7.6	6.3**	8.3	5.5**	0.32	**	**
(%)	150	4.8	3.6**	6.7	4.5**	7.1	6.4**	8.6	6.0**	0.27	**	**
Aerobic stability	60	30	127*	24	153**	31	150**	32	>170**	12.0	**	ns
(h)[d]	150	94	>170**	43	>170**	76	>170**	39	>170**	12.8	**	**

[a] standard error of the mean
[b] significance of treatment effect
[c] significance of interaction between treatment (T) and growth stage (S)
[d] aerobic stability was measured for a maximum of 170 h

ns: not significant ($p > 0.05$), * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$
nd: not determined because of non-parametric Kruskall-Wallis analysis

The pH of the control silage after 60 d of ensiling varied between 4.41 for stage 3 to 4.93 for stage 1. Longer ensiling increased pH with 0.2 to 1.0 units; the greatest increase was observed for stage 1. Treating grass significantly ($p \leq 0.01$) decreased pH at all stages and for both ensiling periods. The effect varied from 0.4 to 2.0 units and was most pronounced for the long ensiling period. The difference decreased with later harvest date.

Lactic acid content of the control silage after 60 d varied between 32 g kg^{-1} DM for stage 1 to 46 g kg^{-1} DM for stage 3 and was lower at each stage after 150 d of ensiling. The inoculant significantly ($p \leq 0.01$) increased lactic acid content for both ensiling periods. The effect clearly decreased with later harvest date.

Acetic acid content of the control grass silage after 60 d varied between 11 g kg^{-1} DM for stages 2, 3 and 4 to 26 g kg^{-1} DM for stage 1. Longer ensiling decreased the content for stage 1 and increased the content for stage 3. The addition of the inoculant significantly ($p \leq 0.01$) increased acetic acid content for both ensiling periods. For stage 1 after 60 d of ensiling no difference was observed.

Butyric acid was not detected in the control nor in the treated silages after 60 d. After 150 d of ensiling butyric acid was present in the control silage of stages 3 and 4, but not in the treated silages.

Propionic acid was not detected in any of the control or treated silages.

The total alcohol content of the control silages after 60 d varied from 27 g kg^{-1} DM for stage 2 to 42 g kg^{-1} DM for stage 4. Longer ensiling increased alcohol content, particularly for stages 1 and 2. Treatment significantly ($p \leq 0.01$) lowered alcohol content; the decrease was most pronounced for stages 1 and 2 after long ensiling.

The ammonia fraction of the control silage after 60 d increased gradually with later harvesting from 4.5% for stage 1 to 8.3% for stage 4. Longer ensiling increased the ammonia fraction a little more except for stage 3 when a small decrease was observed. Treatment with the inoculant significantly ($p \leq 0.01$) decreased the ammonia fraction for all stages and both ensiling periods with 0.7 to 2.8%-units.

Aerobic stability of the control silage after 60 d was fairly constant among stages amounting to about 30 h. Longer ensiling increased aerobic stability in all stages. Treatment with the inoculant significantly ($p \leq 0.01$) improved aerobic stability. After 150 d of ensiling, the silage with the inoculant remained stable at all stages for more than 7 d.

Rumen degradability of NDF and OM

The potentially degradable fraction as well as the degradation rate of NDF in the rumen decreased with later harvest date (Table 4). As a result, the rumen fermentable NDF fraction decreased from 67.6% for the first stage to 52.5% for the last stage. The inoculant had no effect on D_{NDF} nor on kd_{NDF} of the grass silage after 60 d. On the other hand after 150 d of ensiling, treatment increased D_{NDF}, the effect being significant ($p \leq 0.01$) at stages 2 and 4, whereas it decreased kd_{NDF}, but the difference was only significant ($p \leq 0.01$) at stage 2. Treatment had no effect on the rumen degradable NDF fraction at all growth stages and for the two ensiling periods.

Later harvesting decreased the washable OM fraction and the degradation rate of OM and had no clear effect on the potentially degradable OM fraction. As a result the rumen fermentable OM fraction averaged for both ensiling periods decreased from 74.8% for the first stage to 56.5% for the last stage. Treatment did not affect any of the degradation characteristics after 60 d of ensiling. On the other hand after longer ensiling, treatment significantly ($p \leq 0.05$) increased W_{OM} at stages 1 and 2, had no effect on D_{OM} and decreased kd_{OM}, but only significantly ($p \leq 0.05$) at the last stage after 150 d. As a result, treatment had no effect on FOM% after 60 d of ensiling, whereas it increased %FOM at stages 1 and 2 after 150 d of ensiling.

Table 4. The effect of the inoculant (control C versus treatment T) on rumen degradation characteristics of NDF and OM of grass harvested at 4 growth stages and after an ensiling period (EP) of either 60 or 150 d (means of 3 micro-silos)

	EP	Stage 1		Stage 2		Stage 3		Stage 4		SEM[h]	T[i]	TxS[j]
	(d)	C	T	C	T	C	T	C	T			
D_{NDF}[a]	60	92.9	90.4	87.7	86.6	81.9	82.1	78.2	76.0	1.21	ns	ns
(%)	150	89.5	91.2[ns]	86.9	89.7**	81.8	82.9[ns]	77.6	81.6**	0.96	**	ns
kd_{NDF}[b]	60	6.87	6.63	6.51	5.74	3.81	4.06	4.34	4.34	0.257	ns	ns
(% h^{-1})	150	7.76	7.31[ns]	6.36	5.50**	4.47	4.18[ns]	4.70	4.36[ns]	0.282	**	ns
FNDF[c]	60	67.3	65.2	63.0	61.0	53.1	53.9	52.2	50.7	1.30	ns	ns
(%)	150	65.9	66.7[ns]	62.3	62.7[ns]	54.9	54.9[ns]	52.7	54.5[ns]	1.11	*	ns
W_{OM}[d]	60	43.6	44.9	41.6	39.8	30.2	30.3	28.7	27.5	1.43	ns	*
(%)	150	40.6	46.0**	36.5	40.2**	31.1	30.1[ns]	29.2	28.1[ns]	1.29	**	**
D_{OM}[e]	60	51.1	48.9	47.3	49.4	54.6	54.9	53.2	52.6	0.58	ns	*
(%)	150	50.4	48.3	52.4	51.5	53.9	56.1	52.6	56.1	0.55	ns	**
kd_{OM}[f]	60	7.45	7.79	7.35	7.17	4.22	4.58	4.72	5.17	0.302	ns	ns
(% h^{-1})	150	8.96	8.49[ns]	7.10	6.45[ns]	4.78	4.56[ns]	5.11	4.69*	0.356	*	ns
FOM[g]	60	75.4	75.9	70.9	70.2	56.5	57.9	55.9	55.6	1.79	ns	ns
(%)	150	74.1	77.5*	68.5	70.5*	58.8	58.4[ns]	57.1	56.7[ns]	1.65	**	**

[a] potentially degradable NDF fraction
[b] degradation rate of D_{NDF}
[c] rumen fermentable NDF
[d] washable OM fraction
[e] potentially degradable OM fraction
[f] degradation rate of D_{OM}
[g] rumen fermentable OM
[h] standard error of the mean
[i] significance of treatment effect
[j] significance of interaction between treatment (T) and growth stage (S)
ns: not significant ($p > 0.05$), * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$

Discussion

Control silage

The effect of the inoculant was studied with a first cut grass harvested at four distinct growth stages. The evolution in growth stage was clearly reflected by the increase in NDF content (from 344 to 509 g kg^{-1} DM) and the decrease in CP content (from 238 to 132 g kg^{-1} DM) as well as in OMd (from 91.3 to 73.4%) of the control silage. The growth stage had not only an effect on the quantity of NDF but also on its quality. From the rumen degradation characteristics of NDF it appeared that later harvesting resulted in an increase of the undegradable fraction from about 10% at the first stage to more than 20% at the last stage and means a decreasing potentially degradable fraction. Moreover, the degradation rate of the latter almost halved in the period from the first to the last growth stage. Later harvesting also increased the rumen undegradable OM fraction, whereas the potentially degradable OM fraction remained almost constant because of the decrease of the washable OM fraction. This later tendency can be explained by the decrease of sugars and of soluble protein in silage of a later growth stage.

The inoculant was applied to grass wilted at a DM content of about 35%. Wilting grass is already a good measure to improve silage quality because epiphytic lactic acid bacteria are relatively more tolerant to low moisture availability than the vegetative forms of undesirable clostridia (Woolford 1984). According to the latter there is no more

advantage to wilt further than 300−340 g DM kg^{-1}, because from then on oxidation could increase losses. Indeed the control silage of all 4 stages after 60 d of ensiling showed small DM-losses (1.6 to 2.2%), a low pH (4.4 to 4.9), no butyric acid and a low ammonia fraction (4.5 to 8.3%). Despite the proper fermentation characteristics, aerobic stability of the control silage at all stages was low (about 30 h), considering that the target for potential aerobic stability is 7 d (Wilkinson and Davies 2013).

For testing the efficacy of silage additives, EFSA (2006) recommends an ensiling period of 90 d or longer. In this study a shorter period of 60 d was chosen, because it is a common practice that farmers open their silo after 2 months of ensiling in the assumption that silage fermentation is finished. However, comparison of the control silages after 60 and 150 d of ensiling in the present study showed worse quality after longer ensiling at all stages. Compared with 60 d of ensiling, longer ensiling resulted in higher DM-losses (2.4 to 3.3%), a lower DM content, a higher pH, particularly for the first growth stage, a reduced lactic acid content and an increased alcohol content and the presence of butyric acid at stages 3 and 4. These changes were accompanied by a further decrease of sugar content and indicate that the fermentation process was still ungoing after 60 d of ensiling. Considering the low aerobic stability observed after 60 d of ensiling, the oxidation of the control silages during longer ensiling may have been caused by some air ingression through the tape covering the openings in the tube. Another explanation is the higher risk for aerobic deterioration at a density of 180 kg DM per m^3, as applied in our experiment. Such a density also prevails in practice, but is lower than the recommended minimum density of 210 kg DM per m^3 (Wilkinson and Davies 2013).

The effect of the inoculant on chemical composition and silage quality

The number of LAB counted in the inoculated grass silage corresponded fairly well with the number added with the inoculant, whereas the number in the control silages was low to very low. Notwithstanding the proper quality of the control silage after 60 d of ensiling, treatment clearly improved almost all fermentation characteristics. The inoculant decreased weight losses, resulting in a higher DM content of the silage. It increased lactic acid content; the effect was most pronounced for the first two harvest dates. It also increased acetic acid content at all stages except the first. The increase in the production of acids was reflected in a decrease of pH. On the other hand, the addition of the inoculant decreased the formation of alcohols, which is an indication that yeasts and moulds were inhibited, which is also reflected by the better aerobic stability of the treated silage. That the inoculant reduced the activity of undesirable organisms appears also from the lower ammonia fraction, meaning less protein degradation in the silo. Similar effects by inoculating wilted perennial ryegrass (330 g DM kg^{-1}) with *L. buchneri* plus a mixture of *Pediococcus pentosaceus* and *L. plantarum* on pH, lactic acid, the ammonia fraction, DM loss and aerobic stability were observed by Driehuis et al. (2001).

The production of more acetic acid besides more lactic acid proves the activity of the heterofermentative bacterium *L. buchneri* in the inoculant. Acetic acid inhibits yeasts and moulds, which was clearly reflected by the lower alcohol production and better aerobic stability. The higher acid production by the added living Lactobacilli was possible through the fermentation of more sugars. The addition of the inoculant also lowered NDF content of the silage, particularly at the early growth stages. The decrease of NDF content is interesting for the nutritive value of the silage as it should result in a higher feed intake. Indeed, cell wall content highly affects feed intake by contributing to rumen fill (Jung and Allen 1995).

The decrease in NDF content was accompanied by an increase of RNSP content, a measure of complex sugars. This is another indication of the activity of *L. buchneri*, which was shown to produce ferulate esterase (Donaghy et al. 1998). This enzyme seems able to attack young cell walls, but not lignified ones.

The improvement of silage quality by addition of the inoculant was even more pronounced after 150 d of ensiling. This greater effect is rather due to the worse quality of the untreated silage after 150 d of ensiling than to a prolonged effect of the inoculant. The lower contents of CP and crude ash in the DM of the treated grass silage at all stages after both ensiling periods, although small, may be explained by a dilution effect. Indeed, when expressed per kg of silage, CP and ash contents were similar for the control and the treated silage.

The effect of the inoculant on NDF and OM degradability

Because of the presence of *L. buchneri*, which is able to produce ferulate esterase (FE), the studied inoculant is claimed to improve the nutritive value of the resulting grass silage through a better cell wall degradability in the rumen. Weinberg et al. (2004) have shown that lactic acid bacteria consumed with silage enter the rumen and may survive there. Nsereko et al. (2008) examined the activity of 8 FE producing Lactobacilli by in situ incubations and found that they all increased 48 h rumen NDF degradability of perennial ryegrass by 9 to 11%. The studied strains did not affect NDF content of the silage and one strain increased NDF content, whereas we found a decrease of NDF content in the treated silage of growth stages 1 and 2. In their study however, the control grass silage contained 571 g NDF kg^{-1} DM, which is even more than the cell wall content of the last stage in our study. Thus, it agrees with our finding that the inoculant has no effect on cell wall content in silage from older grass. But also Driehuis et al. (2003) found no effect of *L. buchneri*, with or without homofermentative lactic acid bacteria, on NDF content of perennial ryegrass with 438 g NDF kg^{-1} DM, similar to that of the second growth stage in our experiment. In agreement with our results, Van Vuuren et al. (1989) found a decrease of NDF content when herbage was treated with cell wall degrading enzymes; the effect decreased with increasing maturity and DM content of the grass.

In contrast with Nsereko et al. (2008), who found an increase of NDF degradability with the same *L. buchneri* strain as present in the inoculant of our study, we did not find an effect of treatment on the rumen fermentable NDF fraction at any of the growth stages either after 60 or 150 d of ensiling. Considering the clear positive effects on silage fermentation quality, particularly the increase in acetic acid and the better aerobic stability, one may conclude that *L. buchneri* has worked in our experiment. Moreover, it seems that *L. buchneri* has developed FE activity during the ensiling process by degrading easily degradable cell walls, and so leaving cell walls which were more difficult to degrade in the rumen. Similar observations were done by Van Vuuren et al. (1989) treating herbage with cell wall degrading enzymes.

The percentage of rumen FOM is another interesting nutritive parameter because it determines microbial protein production. The inoculant had no effect on %FOM at any growth stage after 60 d of ensiling, but increased %FOM of the silage from the first and second growth stage after 150 d of ensiling. This increase was mainly due to a higher W_{OM} fraction, which can be explained by the increase in fermentation products as well as complex sugars. The treatment effects for rumen FOM are confirmed by those for in vitro OM digestibility. The latter mimics digestibility over the whole digestive tract and treatment increased OMd after 150 d of ensiling with on average 2.3%-units over the four growth stages.

Conclusions

By treating wilted grass with the inoculant more sugars were fermented to lactic and acetic acid, resulting in a lower pH, less DM-losses and protein degradation and a better aerobic stability. The effects on silage quality were more pronounced after 150 d of ensiling than after 60 d, but this was rather due to the worse quality of the control silage than to a prolonged effect of the inoculant. Although wilting grass is a good measure to obtain good quality silage, quality can still further be improved by using the inoculant.

The treatment lowered NDF content of grass harvested at the early growth stages, probably by degrading NDF to complex sugars but had no effect on NDF degradability in the rumen. The inoculant improved rumen fermentability as well as in vitro digestibility of OM after 150 d of ensiling. Postponing harvest and correcting the nutritive value of the silage by applying inoculant additive seems however no option.

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