The effect of *Lactobacillus buchneri* inoculation on the aerobic stability and fermentation characteristics of alfalfa-ryegrass, red clover and maize silage

Eva Wambacq[1], Joos P. Latré[2] and Geert Haesaert[1]

[1] University College Ghent – Associated Faculty of Applied Bioscience Engineering, Valentijn Vaerwyckweg 1, 9000 Ghent, Belgium

[2] University College Ghent – Faculty of Science and Technology, Brusselsesteenweg 161, 9090 Melle, Belgium

e-mail: eva.wambacq@ugent.be

Aerobic spoilage of silages occurs frequently and is undesirable because it reduces both its nutritive and hygienic quality. Silage inoculants containing heterofermentative lactic acid bacteria, like *Lactobacillus buchneri*, have already been proven to improve aerobic stability by augmented production of acetic acid, which inhibits yeasts. In this study, the effect of *L. buchneri* on fermentation characteristics and aerobic stability of alfalfa-ryegrass silage, red clover silage and maize silage was assessed using microsilos. Two dosages, 1×10^5 and 3×10^5 cfu g^{-1} of fresh matter, were compared to untreated control silage. Inoculation with *L. buchneri* clearly altered the fermentation characteristics of alfalfa-ryegrass and red clover silage, resulting in a significantly higher aerobic stability at both dosages. The effects of *L. buchneri* inoculation on maize silage were less clear, but nevertheless the aerobic stability of maize silage inoculated with 1×10^5 cfu g^{-1} of fresh matter was significantly higher compared to the untreated silage.

Key words : silage additive, *Lactobacillus buchneri*, fermentation, heating

Introduction

Aerobic spoilage and heating of ensiled fodder crops occurs frequently (Wilkinson 2005, Stryszewska and Pys 2006). This process is usually initiated by acid-tolerant yeasts and occasionally by acetic acid bacteria when oxygen is able to penetrate between the plant particles, during feed-out or due to damage of the silo coverage (Spoelstra et al. 1988, Woolford 1990, Driehuis et al. 1999). Yeasts and acetic acid bacteria aerobically break down residual water-soluble carbohydrates and organic acids like lactic acid and acetic acid in a first phase of aerobic deterioration (Oude Elferink et al. 2006). This not only lowers the nutritive value of the silage, but also triggers a raise of the silage-pH, allowing growth of opportunistic bacteria and moulds in a second phase of aerobic spoilage (Weinberg and Muck 1996, Scudamore and Livesy 1998, Stryszewska and Pys 2006). Mould development in silages is undesired since inhalation of high concentrations of certain airborne mould spores can affect the respiratory system and cause allergenic reactions in animals and humans, but also because some mould genera can produce mycotoxins during silage conservation (Bui et al. 1994, Auerbach et al. 1998). Furthermore, mould growth decreases the nutritive value of the silage even more and also lowers its palatability (DiConstanzo et al. 1995, Wilkinson 2005). Mould species that regularly have been isolated from silage belong to the genera *Penicillium*, *Aspergillus*, *Mucor*, *Byssochlamys*, *Fusarium*, *Alternaria*, *Geotrichum*, *Monascus*, *Paecilomyces* and *Trichoderma* (Nout et al. 1993, Auerbach et al. 1998, Samson and Frisvad 2004, Samson et al. 2004, O'Brien et al. 2005, Mansfield et al. 2008, Declerck et al. 2009). *Fusarium*, *Aspergillus* and *Penicillium* are the most important mycotoxin producing mould genera. In animals, mycotoxins are factors contributing to chronic problems like a higher incidence of disease and lower production efficiency (Scudamore and Livesey 1998, Barug et al. 2006, Fink-Gremmels 2008).

Application of a silage inoculant consisting of heterofermentative lactic acid bacteria (HeLAB) has already been proven to reduce heating and increase aerobic stability of silages of maize, grass and other fodder crops (Driehuis et al. 1999, Driehuis et al. 2001, Adesogan et al. 2003, Filya et al. 2006, Kleinschmit and Kung 2006). While homofermentative lactic acid bacteria (HoLAB) improve silage fermentation by very efficient production of lactic acid, heterofermentative lactic acid bacteria produce both lactic acid and acetic acid - the latter having an inhibiting effect on yeasts and moulds (Moon 1983, Oude Elferink et al. 2001, Filya et al. 2006). Therefore, HeLAB inoculants should decrease aerobic spoilage of silages (Kung and Ranjit 2001, Danner et al. 2003).

The aim of this study was to assess the effect of HeLAB inoculation with *Lactobacillus buchneri* on aerobic stability and fermentation characteristics of three silage types : red clover (*Trifolium pratense*), whole crop maize (*Zea mays*) and a mixture of alfalfa (*Medicago sativa*) and Italian ryegrass (*Lolium multiflorum*).

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Material and methods

Ensiling trials

In 2008, trials were performed with a mixture of alfalfa and Italian ryegrass (approx. 50/50 w/w) and with red clover, while the trial with whole crop maize was conducted in 2009. Microsilos with a content of 2.75 litre (height 35 cm, diameter 10 cm) equipped with a CO_2-valve were used. The microsilos had two openings (diameter 0.5 cm) covered with plastic tape, to be able to provide aerobic stress by removing the tape.

The alfalfa-ryegrass was from the first cut, the red clover from the second cut. Both crops were harvested at flowering with a Haldrup mower (Inotec, Løgstør, Denmark), spread in a thin layer and wilted to approximately 350 gram dry matter per kilogram fresh matter (quick estimation by microwave drying) during a few hours. Both crops were chopped to approximately 5 cm with a New Holland chopper (Sperry New Holland, Zedelgem, Belgium). The maize variety 'Lafortuna' was chopped to approximately 5 mm with a Mengele chopper (Mengele Agrartechnik, Standort Waltstetten, Germany).

A representative sample of untreated chopped material was taken for determination of dry matter (DM), water-soluble carbohydrates (WSC), crude ash (CA), crude protein (CP), crude fat (CF), *in vitro* digestibility, neutral detergent fibre (NDF) and acid detergent fibre (ADF). The Net Energy for Lactation (NEL) was estimated (formulas in Debrabander 2011). Counts of epiphytic yeasts, moulds, lactic acid bacteria and enterobacteria were also performed. The results of the analyses on the herbage prior to ensiling are summarized in Table 1.

Table 1. Chemical composition, nutritive value and microbial counts of raw materials prior to ensiling

	Alfalfa- ryegrass	Red clover	Maize
Dry matter (g kg^{-1} FM)	383	371	351
Water-soluble carbohydrates (g kg^{-1} FM)	14	26	25
Crude ash (g kg^{-1} DM)	149	95	44
Crude protein (g kg^{-1} DM)	219	209	64
Crude fat (g kg^{-1} DM)	23	14	24
Neutral-detergent fibre (g kg^{-1} DM)	441	516	636
Acid-detergent fibre (g kg^{-1} DM)	258	299	241
Net energy for lactation (MJ)	4.58	4.70	6.53
In vitro digestibility of OM (%)	81.0	69.5	71.2
Yeasts (log cfu g^{-1} FM)	< 2	< 2	5.7
Moulds (log cfu g^{-1} FM)	4.0	4.2	4.8
Lactic acid bacteria (log cfu g^{-1} FM)	6.7	7.0	5.5
Enterobacteria (log cfu g^{-1} FM)	< 1	< 1	4.8

FM: fresh matter, DM: dry matter, MJ: mega-Joule, OM: organic matter, cfu: colony-forming units

Silages were inoculated with *L. buchneri* NCIMB 40788 (Lallemand sas). In each trial, the following treatments were included: a control treatment (C) of sterile demineralized water, a low dosage of *L. buchneri* (LD) and a high dosage of *L. buchneri* (HD). LD-solution was dosed at 1×10^5 cfu g^{-1} of fresh matter, while the dosage of the HD-solution was 3×10^5 cfu g^{-1} of fresh matter. On the *L. buchneri* inoculant powder, counting of lactic acid bacteria was performed prior to ensiling to ensure correct dosage. Inoculants were suspended in sterile demineralized water with 0.85% sodium chloride just before ensiling. All solutions were applied homogeneously on chopped material by hand held sprayers in a ratio of 10 ml $kg^{-1}$of fresh matter. A different sprayer was used per treatment. Six microsilos were ensiled per treatment, at a mean silo density of respectively 165, 154 and 196 kg DM per cubic meter for alfalfa-ryegrass, red clover and maize silage. Microsilos were weighed empty and immediately after filling, which was continued on a weekly basis. All microsilos were subjected to aerobic stress during 24 hours at 71–72 days after ensiling. At silo opening after 90 days, samples were taken from five microsilos per treatment for chemical analyses (dry matter, crude protein, ammonia, ethanol, pH, lactic acid, acetic acid, butyric acid and propionic acid), microbial analyses (counts of yeasts, moulds and lactic acid bacteria) and determination of the aerobic stability.

Chemical analyses

DM content was determined by air drying at 65 °C during 170 hours. On dried material, WSC content was analysed by the Luff-Schoorl method, while CA, CP, CF, *in vitro* digestibility, NDF and ADF were determined according to ISO 17025 as follows: CA by 71/250/EG, CP by the Dumas combustion method according to NF ISO 15670 and CF by 71/393/EG (European Economic Community 1971), *in vitro* digestibility according to De Boever et al. (1986), NDF and ADF according to Van Soest et al. (1991). Fresh silage material was stored at –20 °C immediately after sampling for subsequent analyses. Ethanol content was determined on an aqueous extract of fresh material by NIR absorption by an adaptation of the method described by Sørensen (2004). Measurement of pH was done on a 1/10 (w/w) aqueous extract of fresh material (Muck et al. 1999). Also on fresh material, ammonia and crude protein were determined according to Kjeldahl (1883), while lactic acid, acetic acid, butyric acid and propionic acid were determined by HPLC (Ohmomo et al. 1993). DM content at silo opening was corrected (indicated as "cDM") for volatile compounds (Dulphy and Demarquilly 1981).

Microbial counts

Samples were stored at 4 °C prior to counting, which was started within one day after sampling. Counts of lactic acid bacteria and yeasts and moulds were conducted according to resp. ISO 15214 (1998) and ISO 21527-1 (2008), while enterobacteria were counted with Petrifilm® Afnor 3M 01/6-09/97.

Aerobic stability

The aerobic stability was determined based on the protocol suggested by Honig (1990). The equivalent of 100 g DM was placed loosely into polystyrene boxes and allowed to deteriorate aerobically at 20 °C ± 1 °C. The top and bottom of the boxes had an opening to allow gas exchange. Per microsilo, two polystyrene boxes were filled. A double layer of cheesecloth allowing air penetration was placed over each container to prevent drying. A thermocouple probe was placed into the geometric centre of each silage mass. Silage temperature was recorded every 10 minutes and averaged over 2-hour periods. Silages were not disturbed during testing. Monitoring was carried out over a period of 7 days for the maize silage and of 15 days for the alfalfa-ryegrass and red clover silages. An increase of silage temperature of 3 °C above surrounding temperature was taken as cut-off for aerobic stability.

Statistical analyses

Data were statistically analyzed with SAS 4.1 (SAS 2006). Normality was tested by Kolmogorov-Smirnov, while equality of variances was checked by Levene's test. Normally distributed, homoscedastic data were subjected to one-way ANOVA with Tukey as *post hoc* test. Otherwise, data were subjected to non-parametric one-way ANOVA with Wilcoxon as *post hoc* test, applying Bonferroni correction. Significance was declared at $p < 0.05$, with p-values of < 0.001 marked as "***", < 0.01 as "**" and < 0.05 as "*". Non-significance was indicated as "n.s.".

Results

Fermentation losses

Fermentation losses were calculated based on the fresh weight losses of the microsilos during the ensiling period. The evolution of the fermentation losses is presented in Table 2 (five observations per treatment for each silage type).

Table 2. Evolution of fermentation losses (g kg^{-1} fresh matter) during ensiled period

Fermentation losses (g kg^{-1} FM) after		Alfalfa-ryegrass				Red clover				Maize		
		mean	SD	sign.		mean	SD	sign.		mean	SD	sign.
7 days	C	4.45	0.13	a	***	5.39	0.24		n.s.	7.28	0.19	n.s.
	LD	8.07	0.32	b		5.69	0.54			7.19	0.32	
	HD	8.41	0.34	b		5.94	0.74			7.22	0.09	
14 days	C	6.17	0.10	a	***	6.54	0.32	a	***	7.88	0.34	n.s.
	LD	10.7	0.30	b		7.98	0.64	b		8.03	0.70	
	HD	10.8	0.29	b		8.17	0.85	b		7.97	0.20	
21 days	C	6.68	0.23	a	***	7.26	0.33	a	***	8.33	0.42	n.s.
	LD	11.6	0.34	b		8.96	0.67	b		8.56	0.84	
	HD	11.6	0.15	b		9.16	1.00	b		8.54	0.22	
28 days	C	7.12	0.22	a	***	7.42	0.42	a	***	8.66	0.45	n.s.
	LD	12.2	0.33	b		9.33	0.81	b		9.00	0.79	
	HD	12.4	0.19	b		9.44	1.16	b		8.95	0.26	
35 days	C	7.47	0.16	a	***	7.65	0.41	a	***	8.83	0.55	n.s.
	LD	12.8	0.43	b		9.59	0.79	b		9.13	0.78	
	HD	12.9	0.19	b		9.77	1.12	b		9.13	0.24	
42 days	C	8.11	0.27	a	***	8.05	0.51	a	***	8.97	0.63	n.s.
	LD	13.6	0.41	b		10.1	0.84	b		9.31	0.82	
	HD	13.7	0.21	b		10.4	1.17	b		9.34	0.24	
49 days	C	8.52	0.22	a	***	8.19	0.44	a	***	9.07	0.66	n.s.
	LD	14.0	0.35	b		10.3	0.85	b		9.43	0.81	
	HD	14.1	0.21	b		10.5	1.29	b		9.48	0.22	
56 days	C	8.70	0.27	a	***	8.26	0.42	a	***	9.16	0.68	n.s.
	LD	14.2	0.34	b		10.4	0.87	b		9.51	0.81	
	HD	14.3	0.17	b		10.7	1.32	b		9.60	0.20	
63 days	C	9.36	0.25	a	***	8.46	0.32	a	***	9.32	0.70	n.s.
	LD	14.9	0.34	b		10.8	0.91	b		9.66	0.87	
	HD	15.0	0.17	b		11.0	1.32	b		9.73	0.22	
70 days	C	9.90	0.27	a	***	8.67	0.47	a	***	9.40	0.72	n.s.
	LD	15.4	0.31	b		10.9	0.98	b		9.77	0.86	
	HD	15.5	0.17	b		11.2	1.33	b		9.84	0.20	
aerobic stress provided at 71–72 days after ensiling												
77 days	C	11.0	0.34	a	***	9.21	0.46	a	***	10.5	0.32	n.s.
	LD	15.9	0.10	b		11.8	0.59	b		10.5	0.52	
	HD	16.0	0.16	b		12.0	1.04	b		10.4	0.13	
84 days	C	11.0	0.34	a	***	9.23	0.48	a	***	10.5	0.30	n.s.
	LD	15.9	0.10	b		11.8	0.59	b		10.6	0.59	
	HD	16.0	0.16	b		12.0	1.04	b		10.5	0.13	
90 days	C	11.2	0.38	a	***	9.41	0.41	a	***	10.6	0.26	n.s.
	LD	16.0	0.09	b		12.0	0.61	b		10.7	0.47	
	HD	16.1	0.15	b		12.2	1.10	b		10.7	0.12	

SD: standard deviation, sign.: significance, n.s.: not significant, ***: $p < 0.001$
FM: fresh matter
C: control
LD: low dosage of L. buchneri (1×10^5 cfu g^{-1} of fresh matter)
HD: high dosage of L. buchneri (3×10^5 cfu g^{-1} of fresh matter)

For the alfalfa-ryegrass silage inoculated with *L. buchneri*, either at high or low dosage, the fermentation losses were significantly higher compared to the untreated control. A similar pattern was detected in the red clover silage from 14 days after ensiling on till the end of the ensiling period. High dosage of *L. buchneri* resulted in slightly higher fermentation losses than low dosage, but the differences between low dosage and high dosage of *L. buchneri* were not significant. No significant differences in fermentation losses between treatments were observed for the maize silage.

Fermentation characteristics

Based on the water-soluble carbohydrate content of the herbage prior to ensiling, the alfalfa-ryegrass mixture was considered as difficult to ensile (< 15 g kg^{-1} fresh matter of water-soluble carbohydrates) according to the EFSA opinion on silage additives guidelines (European Food Safety Authority 2008), while the red clover and whole crop maize were considered to be moderately easy to ensile (15–30 g kg^{-1} fresh matter of water-soluble carbohydrates). Due to the evidently higher crude protein content of the alfalfa-ryegrass silage and red clover silage, a higher buffering capacity was expected for these silages compared to maize silage (McDonald et al. 1991, Wilkinson 2005).

The fermentation characteristics of the silages are listed in Table 3 (five observations per treatment for each silage type).

Table 3. Effect of *Lactobacillus buchneri* inoculation on fermentation characteristics of alfalfa-ryegrass, red clover and maize silage

		Alfalfa-ryegrass				Red clover				Maize			
		mean	SD	sign.		mean	SD	sign.		mean	SD	sign.	
cDM (g kg^{-1} FM)	C	419	3.3	n.s.		371	2.0	a	*	356	2.6	a	
	LD	417	4.2			369	4.9	ab		344	3.1	b	***
	HD	421	4.3			364	4.4	b		349	1.8	c	
Crude protein (g kg^{-1} cDM)	C	209	4.2			198	4.7	n.s.		60.6	1.02		
	LD	212	1.7	a	*	199	7.5			61.5	1.15	n.s.	
	HD	205	3.1	b		191	4.7			59.7	0.95		
Ammonia (g kg^{-1} cDM)	C	5.79	0.1	a		5.86	0.28	n.s.		0.69	0.05		
	LD	6.31	0.1	b	***	5.96	0.29			0.73	0.04	n.s.	
	HD	6.21	0.2	b		5.89	0.26			0.75	0.03		
Ammonia nitrogen (g kg^{-1} nitrogen)	C	138	3.9	a		145	7.7	n.s.		52.3	3.1	a	
	LD	148	2.8	b	***	148	7.1			54.1	2.7	ab	*
	HD	151	3.4	b		152	8.8			57.2	2.4	b	
Ethanol (g kg^{-1} cDM)	C	17.3	1.2	a		18.5	3.30	n.s.		19.7	3.11		
	LD	21.6	0.4	b	***	19.7	0.66			20.9	2.72	n.s.	
	HD	21.7	1.3	b		20.0	2.32			21.2	1.45		
pH	C	4.54	0.01	a		4.36	0.02	a		3.77	0.01	ab	
	LD	4.58	0.01	b	***	4.46	0.02	b	***	3.77	0.02	a	*
	HD	4.59	0.02	b		4.47	0.06	b		3.74	0.01	b	
Lactic acid (g kg^{-1} cDM)	C	48.3	1.32	a		64.9	1.44	a		41.3	3.06	a	
	LD	41.2	2.48	b	**	56.0	3.49	ab	*	45.0	2.38	b	*
	HD	40.8	4.27	b		50.9	11.3	b		45.4	1.71	b	
Acetic acid (g kg^{-1} cDM)	C	18.5	2.99	a		22.1	1.06	a		15.9	2.51	a	
	LD	30.2	3.70	b	***	30.7	3.66	ab	*	19.5	2.55	ab	**
	HD	31.1	4.06	b		34.1	9.46	b		21.3	1.58	b	
Butyric acid (g kg^{-1} cDM)	C	0.12	0.27	n.s.		0.00	0.00	-		0.00	0.00		
	LD	0.00	0.00			0.00				0.00	0.00	-	
	HD	0.00	0.00			0.00				0.00	0.00		

SD: standard deviation, sign.: significance, n.s.: not significant, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$
C: control
LD: low dosage of *L. buchneri* (1×10^5 cfu g^{-1} of fresh matter)
HD: high dosage of *L. buchneri* (3×10^5 cfu g^{-1} of fresh matter)
FM: fresh matter
cDM: corrected DM content, according to Dulphy and Demarquilly (1981)

The corrected dry matter content of the silages did not differ significantly between treatments for alfalfa-ryegrass silage, however inoculation with *L. buchneri* lowered the DM content of red clover and maize silage compared to untreated control silage. In case of red clover silage, a significant difference in DM content between the untreated control (371 g kg^{-1} FM) and the high dosage of *L. buchneri* (364 g kg^{-1} FM) was observed, while for maize silage the DM content of all treatments (C : 356, LD : 344, HD : 349 g kg^{-1} FM) differed significantly.

The crude protein content of the alfalfa-ryegrass silage and the red clover silage was within normal range, while the maize silage was relatively low in crude protein (Hoffman and Broderick 2001, Debrabander 2011). No significant differences in crude protein content between treatments were observed in red clover silage and maize silage. The crude protein content of alfalfa-ryegrass silage treated with low dosage of *L. buchneri* (212 g kg^{-1} cDM) was significantly higher compared to high dosage (205 g kg^{-1} cDM), while the untreated control silage had an intermediate crude protein content (209 g kg^{-1} cDM).

As expected, the ammonia content of maize silage was lower than the ammonia content of alfalfa-ryegrass silage and red clover silage. Red clover silage and maize silage showed no significant differences in ammonia content between treatments. The ammonia content of *L. buchneri* inoculated alfalfa-ryegrass silage (LD: 6.31, HD: 6.21 g kg^{-1} cDM) was significantly higher than that of untreated silage (5.79 g kg^{-1} cDM), and the same observation can be made for the ammonia nitrogen fraction (C: 138, LD: 148, HD: 151 g ammonia nitrogen kg^{-1} total nitrogen). The fraction of ammonia nitrogen to total nitrogen of untreated maize silage (52.3 g ammonia nitrogen kg^{-1} total nitrogen) was significantly lower compared to maize silage with high dosage of *L. buchneri* (57.2 g ammonia nitrogen kg^{-1} total nitrogen), while no significant differences in ammonia nitrogen fraction between treatments were observed in red clover silage.

In all three silage types, the ethanol content was lower in untreated silage compared to *L. buchneri* inoculated silage. These differences between treatments were only significant in alfalfa-ryegrass silage (C: 17.3, LD 21.6, HD :21.7 g kg^{-1} cDM).

Looking at the pH values, it can be noticed that the maize silage had clearly lower pH values than the alfalfa-ryegrass silage and the red clover silage - the latter two silage types having a higher buffering capacity due to a higher crude protein content. In alfalfa-ryegrass silage, inoculation with *L. buchneri* at both low and high dosage (LD: 4.58, HD: 4.59) resulted in a significantly higher pH compared to untreated control silage (4.54). The same pattern was observed in red clover silage (C: 4.36, LD: 4.46, HD: 4.47). In maize silage, the lowest pH values were observed after inoculation with high dosage of L. *buchneri* (3.74), which resulted in a significantly lower pH than after inoculation with low dosage of *L. buchneri* (3.77); the untreated control silage had an intermediate pH (3.77). It should be noted that in absolute value the differences between treatments were low for all three silage types.

The alfalfa-ryegrass silage inoculated with *L. buchneri* at low dosage and at high dosage had a significantly lower lactic acid content (LD: 41.2, HD: 40.8 g kg^{-1} cDM) and a significantly higher acetic acid content (LD: 30.2, HD: 31.1 g kg^{-1} cDM) than untreated control silage (lactic acid: 48.3 - acetic acid: 18.5 g kg^{-1} cDM). Butyric acid was only detected in untreated alfalfa-ryegrass silage; not in the red clover and maize silages. High dosage of *L. buchneri* in red clover silage resulted in a significantly lower lactic acid content (50.9 g kg^{-1} cDM) and significantly higher acetic acid content (34.1 g kg^{-1} cDM) compared to no inoculation (lactic acid: 64.9, acetic acid: 22.1 g kg^{-1} cDM). Red clover silage inoculated with low dosage of *L. buchneri* contained intermediate levels of lactic acid (56.0 g kg^{-1} cDM) and acetic acid (30.7 g kg^{-1} cDM). In maize silage, significantly higher lactic acid levels were observed after inoculation with *L. buchneri* (LD: 45.0, HD: 45.4 g kg^{-1} cDM). Also acetic acid level was increased significantly after inoculation with high dosage of *L. buchneri* (21.3 g kg^{-1} cDM) compared to untreated maize silage (15.9 g kg^{-1} cDM).

Microbial counts and aerobic stability

The results of the microbial counts at silo opening and of the aerobic stability determination are presented in Table 4 (five observations per treatment for each silage type).

In all three silage types, yeast counts in untreated silage at silo opening were slightly higher compared to inoculated silage, but the differences were not significant.

No significant differences in mould counts between treatments were observed in red clover silage and maize silage, but *L. buchneri* inoculated alfalfa-ryegrass silage contained significantly less mould spores (LD: 2.03, HD: 2.21 log cfu g^{-1} FM) than untreated silage (3.25 log cfu g^{-1} FM).

Table 4. Effect of *Lactobacillus buchneri* inoculation on microbial counts and aerobic stability of alfalfa-ryegrass, red clover and maize silage

		Alfalfa-ryegrass			Red clover			Maize		
		mean	SD	sign.	mean	SD	sign.	mean	SD	sign.
Yeasts (log cfu g^{-1} FM)	C	2.03	0.15	n.s.	2.58	0.57		3.10	1.17	
	LD	1.96	0.02		2.06	0.23	n.s.	2.72	1.72	n.s.
	HD	1.95	0.00		2.26	0.69		2.31	0.51	
Moulds (log cfu g^{-1} FM)	C	3.25	1.00	a	2.21	0.58	n.s.	1.95	0.00	
	LD	2.03	0.15	b *	2.27	0.66		2.17	0.49	n.s.
	HD	2.21	0.22	b	1.97	0.03		2.21	0.57	
Lactic acid bacteria (log cfu g^{-1} FM)	C	6.93	0.28	n.s.	6.78	0.62	a ***	6.59	0.35	a
	LD	7.17	0.24		8.21	0.45	b	7.36	0.24	b ***
	HD	7.24	0.28		7.90	0.31	b	7.41	0.22	b
Aerobic stability (hours)	C	145	97.2	a	296	22.2	a	85.9	15.6	a
	LD	360°	0.0	b ***	360°	0.0	b ***	127	28.7	b *
	HD	360°	0.0	b	360°	0.0	b	115	16.0	ab

SD: standard deviation, sign.: significance, n.s.: not significant, ***: $p < 0.001$, *: $p < 0.05$
C: control
LD: low dosage of *L. buchneri* (1×10^5 cfu g^{-1} of fresh matter)
HD: high dosage of *L. buchneri* (3×10^5 cfu g^{-1} of fresh matter)
cfu: colony-forming units
FM: fresh matter
° no heating within 15 days after desiling: aerobic stability of 360 hours as fixed value

Inoculation with *L. buchneri* at ensiling elevated the lactic acid bacteria numbers, with the differences between inoculated treatments and untreated control being significant for red clover silage (C: 6.78, LD: 8.21, HD : 7.90 log cfu g^{-1} FM) and maize silage (C: 6.59, LD: 7.36, HD: 7.41 log cfu g^{-1} FM).

In alfalfa-ryegrass silage (C: 145, LD: 360, HD: 360 h) and red clover silage (C: 296, LD: 360, HD: 360 h), inoculation with *L. buchneri* significantly increased the aerobic stability at both dosages. In these two silage types, inoculated silage did not heat up to 3 °C above surrounding temperature within 15 days of aerobic stability testing. The aerobic stability of maize silage was clearly lower compared to alfalfa-ryegrass silage and red clover silage. The untreated maize silage (85.9 h) was significantly less stable than the maize silage inoculated with low dosage of *L. buchneri* (127 h). High dosage of *L. buchneri* resulted in maize silage with intermediate aerobic stability (115 h).

Discussion

Lactobacillus buchneri belongs to the heterofermentative lactic acid bacteria and thus exerts the HeLAB metabolism: hexose and pentose sugars are converted into lactic acid, acetic acid, carbon dioxide, ethanol etc. (Kandler 1983, McDonald et al. 1991). HeLAB are less efficient in producing lactic acid than HoLAB, usually resulting in higher fermentation losses after HeLAB inoculation (Driehuis et al. 1999, Filya et al. 2006). This was confirmed statistically in the trials with alfalfa-ryegrass silage and red clover silage, and the same trend could be remarked for the maize silage. Aerobic stress was provided to all microsilos at 71–72 days after ensiling. Ingression of air caused secondary fermentations, which further increased the fermentation losses (Woolford 1990).

The expected effects of HeLAB inoculation on the fermentation characteristics of silage are higher DM losses during the ensiling period, production of less lactic acid and more acetic acid, higher pH and higher ethanol compared to untreated silage (Driehuis et al. 2001, McDonald et al. 1991, Filya et al. 2006, Oude Elferink et al. 2006). Since *L. buchneri* is a slow-growing bacterial species, the pH might drop slowly in *L. buchneri* inoculated silage, allowing enterobacteria more time for protein breakdown, possibly resulting in elevated ammonia levels and higher ammonia nitrogen fraction (Driehuis et al. 2001). The elevated acetic acid levels slow down the first phase of aerobic deterioration by inhibiting yeasts and thus increase aerobic stability. Lower yeast counts are expected after inoculation with HeLAB, which might reduce also mould development in the second phase of aerobic spoilage (Moon 1983, Lindgren et al. 1985, Driehuis et al. 1999). Many of these expectations were confirmed statistically in the ensiling trials that were performed.

The corrected dry matter content of the silage was significantly higher in untreated red clover silage than in red clover silage inoculated with high dosage of *L. buchneri*. In maize silage, inoculation with *L. buchneri* resulted in significantly lower dry matter contents both at low and high dosage. It should however be noted that these differences in mean dry matter content are relatively small, with high standard deviations. Ammonia contents were systematically higher in *L. buchneri* inoculated silage for all three silage types, but this was confirmed statistically only in the alfalfa-ryegrass silage. Ammonia nitrogen fraction followed the same trend as ammonia content, being significantly higher after inoculation with *L. buchneri* for alfalfa-ryegrass silage at both low and high dosage and significantly lower for untreated maize silage compared to maize silage inoculated with high dosage of *L. buchneri*. The alfalfa-ryegrass silage and the red clover silage had a high ammonia nitrogen content, reflecting a moderate to poor silage conservation quality. The maize silage on the other hand was conserved well based on its low ammonia nitrogen fraction (Wilkinson 2005). These observations are in accordance with the expectations based on the chemical composition of the raw material. On the basis of the water-soluble carbohydrate content, the alfalfa-ryegrass mixture was considered to be difficult to ensile, while the red clover silage and maize silage should be moderately easy to ensile. Moreover, the protein content of red clover silage was much higher compared to maize silage, which should result in a higher buffering capacity. The higher conservation quality of maize silage compared to the other two silage types was thus expected. Ethanol content of untreated silage was systematically lower than ethanol content of *L. buchneri* inoculated silage; this difference was significant for alfalfa-ryegrass silage.

It should be noted that for all three silage types the pH values did not differ much in absolute value and the pH values reflected a good silage conservation (Wilkinson, 2005). Inoculation with *L. buchneri*, either at low or high dosage, resulted in significantly higher pH values in alfalfa-ryegrass silage and red clover silage. The significantly higher pH values after inoculation in alfalfa-ryegrass silage and red clover silage can be attributed to lower lactic acid (pK_a 3.85) contents in combination with higher acetic acid (pK_a 4.75) levels of inoculated silage compared to untreated control silage. Contradictory to the expectations, these trends were not observed likewise in maize silage: *L. buchneri* inoculated silage did contain more acetic acid than untreated control silage, but also more lactic acid. This could be attributed to the characteristics of the epiphytic lactic acid bacteria, which were not determined. Only inoculation of maize silage with high dosage of *L. buchneri* resulted in significantly higher acetic acid levels compared to untreated control. The lowest pH was observed in maize silage inoculated with high dosage of *L. buchneri*, the highest pH in maize silage inoculated with low dosage of *L. buchneri*. Furthermore, the aerobic stability of the maize silage after inoculation with low dosage of *L. buchneri* was significantly higher compared to the untreated control, with the high dosage of *L. buchneri* having an intermediate aerobic stability. This observation was quite surprising, regarding the non-significant difference in acetic acid content between maize silage inoculated with low dosage of *L. buchneri* and the untreated control silage. In none of the three silage types studied inoculation with *L. buchneri* resulted in significantly lower yeast counts, but the absolute numbers of yeasts were lower after inoculation. For red clover silage and maize silage, mould counts did not differ either between inoculated silage and control silage. Alfalfa-ryegrass silage did contain less moulds after inoculation with *L. buchneri* at both dosages.

Differences between the two dosages of *L. buchneri* in fermentation quality and aerobic stability of the three silage types were rather limited. High dosage of *L. buchneri* compared to low dosage did not result in significant differences in fermentation losses, pH at silo opening and aerobic stability for the alfalfa-ryegrass and red clover silage. For alfalfa-ryegrass, both *L. buchneri* dosages did not differ significantly in ammonia content, ammonia fraction, ethanol content, lactic acid content and acetic acid content. However for red clover silage, only high dosage of *L. buchneri* caused a significantly lower lactic acid and higher acetic acid level compared to untreated control silage. Driehuis et al. (2001) found in one experiment that *L. buchneri* type activity was higher in case of inoculation with 1×10^5 cfu g^{-1} FM compared to 3×10^5 cfu g^{-1} FM. This was attributed to augmented competitiveness of *L. buchneri* towards epiphytic lactic acid bacteria at lower DM content (approx. 255 g kg^{-1} FM) than at higher DM content (approx. 366 g kg^{-1} FM). In the three ensiling trials performed, the DM content at ensiling was 383, 371 and 351 g kg^{-1} FM for alfalfa-ryegrass, red clover and maize silage, respectively. These values are near the higher DM content as referred to by Driehuis et al. (2001), so it should not be surprising that only few significant differences were observed between high and low dosage of *L. buchneri* in these ensiling trials.

Conclusion

Overall, it can be concluded that inoculation with the HeLAB *L. buchneri* did succeed in altering the silage fermentation characteristics, resulting in better aerobic stability. The effect is however less clear in maize silage, which typically has a good ensilability, than in alfalfa-ryegrass and red clover silages, which have a higher buffering capacity due to a higher crude protein content.

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