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Effect of lactic acid bacteria inoculants, formic acid, potassium sorbate and sodium benzoate on fermentation quality and aerobic stability of wilted grass silage

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The efficiency of a novel strain of lactic acid bacteria inoculant (*Lactobacillus plantarum* VTT E-78076, E76) on the fermentation quality of wilted silage was studied. Furthermore, the possibility to improve aerobic stability of silages by combining an inoculant and chemical preservatives was investigated. Two experiments were conducted with wilted timothy-meadow fescue herbage (dry matter 429 and 344 g kg^{-1}) using six treatments. In experiment I, E76 (10^6 cfu g^{-1} fresh matter (FM)) was applied alone and in combination with sodium benzoate (0.3 g kg^{-1} grass FM) or low rate of formic acid (0.4 l t^{-1} FM). In experiment II, E76 and a commercial inoculant were applied alone and in combination with sodium benzoate. Untreated silage and formic acid (4 l t^{-1} FM) treated silage served as negative and positive controls in both experiments. The effect of sodium benzoate and potassium sorbate in experiment I, on aerobic stability was tested by treating silages prior to aerobic stability measurements. The novel lactic acid bacteria inoculant was equally effective in improving fermentation quality as the commercial inoculant. However, the aerobic stability of both inoculated silages was poorer than that of formic acid treated or the untreated one in one of the experiments. The results suggested that antimicrobial properties of E76 were not effective enough to improve aerobic instability. One option to overcome this problem is to use chemical additives in combination with the inoculants.

Key words: silage making, grass silage, wilting, additives, aerobic stability, inoculants, formic acid, fermentation, sodium benzoate, potassium sorbate

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Introduction

Biological and chemical silage additives are used primarily to improve the fermentation quality and are therefore considered more useful in low dry matter (DM) silage. Direct acidification with an organic acid, for example with formic acid, restricts fermentation i.e increases residual water soluble carbohydrates (WSC) and decreases production of volatile fatty acids (VFA) and protein degradation (Chamberlain and Quig 1987, Jaakkola et al. 1991, 2006). The improved fermentation quality increases silage intake, microbial protein synthesis in the rumen and animal production (Huhtanen et al. 2002, 2003). Increasing the DM content of the herbage by wilting restricts the silage fermentation due to the lower water activity.

Lactic acid bacteria (LAB) inoculants can be used to improve the silage fermentation quality instead of corrosive and hazardous acid based additives. *Lactobacillus plantarum* (VTT E-78076, E76) is known for its broad-spectrum antimicrobial activity both against gram positive and gram negative bacteria and, furthermore, against *Fusarium* moulds (Haikara et al. 1993, Haikara and Laitila 1995, Niku-Paavola et al. 1999, Laitila et al. 2002). Despite being originally isolated from beer, the *L. plantarum* E76 strain proved to be a potential grass silage inoculant in a screening procedure (Skyttä et al. 2002a) and in a laboratory ensiling trial (Saarisalo et al. 2006). It was efficient in producing lactic acid, lowering pH rapidly and decreasing the ammonia-N production. Although efficient in improving silage fermentation, homofermentative LAB inoculants have sometimes impaired the aerobic stability (Weinberg et al. 1993, Weinberg and Muck 1996).

Increasingly herbage is wilted prior to ensiling in northern Europe mainly due to technological advantages. Dryer herbage decreases risk of poor fermentation quality estimated by the amount of VFA and proportion of ammonia-N of nitrogen (Jonsson et al. 1990, Yan et al. 1998, Field et al. 1999). However, with increasing DM, problems associated with aerobic stability tend to increase (Wyss 1999). Yeasts capable of utilising lactate as the source of energy start the aerobic deterioration (Woolford 1990, McDonald et al. 1991). The decrease in lactate increases the silage pH which improves growth conditions for the microbes, deteriorating the nutritional and hygienic quality of silage. The aerobic catabolism of nutrients generates heat, and therefore, an increase in silage temperature is a good indicator for the growth of microbes and nutrient losses. Although the role of additives in controlling fermentation is less important in wilted material, there might be an additional benefit in using additives in high DM silages in order to improve aerobic stability.

Hurdle technology could provide an alternative tool for controlling growth of yeasts in silage since their growth cannot be inhibited by any single environmental parameter. Preservation by hurdle technology is based on simultaneous application of various antimicrobial factors affecting the target organisms by different mechanisms (Leistner and Gorris 1995). In ensiling, either a combination of synergistically working inoculants (Rauramaa et al. 1996, Driehuis et al. 1999, Weinberg et al. 1999), or a combination of an appropriate inoculant and chemical treatment (Beck 1989 ref. by Weissbach et al. 1991) could be considered. Skyttä et al. (2002b) showed that a combination of a selected lactic inoculant, potassium sorbate and sodium benzoate inhibited *in vitro* the growth of four spoilage yeast strains isolated from grass silage [*Pichia anomala* (lactate+) VTT C-00352, *Torulaspora delbrueckii* (lactate+) VTT C-00355, *Rhodotorula mucilaginosa* (lactate-) VTT C-00353 and *Pichia kluyveri* var. *kluyeveri* (lactate-) VTT C-00354].

The first aim of the experiments was to study the effectiveness of the LAB inoculant E76, previously selected (Skyttä et al. 2002a) and tested in the laboratory environment (Saarisalo et al. 2006), in less controlled i.e. more practical and demanding conditions, and to compare it with a commercial LAB inoculant. Another object was to study the possibilities to improve the aerobic stability of wilted silage by applying the hurdle technology. The preliminary results of experiment I have been presented by Saarisalo et al. (2001).

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Material and methods

Material and treatments

Two trials were carried out at MTT Agrifood Research Finland, Jokioinen, using pilot scale silos (1 m^3) made of metallic cylinders and lined with plastic. Both experiments consisted of six treatments (Table 1). In experiment I, silages were prepared from second cut timothy (*Phleum pratense*) -meadow fescue (*Festuca pratensis*) grass, mown with a mower conditioner, wilted for 6 h and lifted with a precision-chop forage harvester. Treatments were: 1) Untreated (UT), 2) Formic acid 4 l t^{-1} FM, [5 l t^{-1} AIV2Plus (formic acid 760 g kg^{-1}, ammonium formate 55 g kg^{-1}, Kemira Oyj, Finland), FA4], 3) *L. plantarum* VTT E-78076 inoculant, freshly cultured, 1×10^6 cfu g^{-1} FM (E76), 4) E76 + a low rate of formic acid (AIV2Plus, 0.4 l t^{-1}, FA0.4), (E76 + FA0.4), 5) E76 + sodium benzoate (J.T.Baker, Deventer, the Netherlands, 0.3 g kg^{-1} FM, E76 + NaB), 6) E76 + FA0.4 + NaB. Additives FA4 and E76 were applied in the field during harvesting of the grass with the precision chopper. During the filling of the silos, sodium benzoate and FA0.4 were applied as a water solution (20 ml kg^{-1} FM) into E76-treated herbage, with an equal amount of tap water into UT herbage.

In experiment II, herbage was first cut timothy-meadow fescue wilted for 6 h. The treatments consisted of 1) No additive (UT), 2) Formic acid 4 l t^{-1} FM (5 l t^{-1} AIV2Plus, FA), 3) *L. plantarum* VTT E-78076 inoculant, freshly cultured, 1×10^6 cfu g^{-1} (E76), 4) E76 + sodium benzoate 0.3 g kg^{-1} (E76 + NaB), 5) Commercial inoculant [AIV Biostart, *L. plantarum* (DSM 4409), BS] and 6) BS + sodium benzoate (BS + NaB). Treatments 2, 3 and 5 were applied to herbage in the field when harvested with the precision chopper while sodium benzoate was added in water solution to treatments 4 and 6 during silo filling.

In both experiments, three replicates for each treatment were prepared as three batches. From the herbages treated in the field, samples were collected for each batch (experiment I: UT, FA4, E76 and in experiment II: UT, FA, E76 and BS). The E76 sample in experiment I represents treatments

Table 1. Silage additives and post-opening treatments in the experiments I and II.

Treatment	Silage additives	Post opening treatments, g kg^{-1} fresh matter
Experiment I		
UT	Untreated	NaB 0.15, 0.30, 0.45
FA4	Formic acid, 4 l t^{-1} FM	KS 0.15, 0.30, 0.45
E76	*L. plantarum* VTT E-78076, 1*10^6 cfu g^{-1} FM	NaB+KS 0.15, 0.30, 0.45
E76+FA0.4	E76+Formic acid, 0.4 l t^{-1} FM	
E76+NaB	E76+Na-benzoate, 0.3 g kg^{-1} FM	
E76+FA0.4+NaB	E76+Na-benzoate, 0.3 g kg^{-1} FM+FA 0.4 l t^{-1}FM	
Experiment II		
UT	Untreated	NaB 0.30
FA	Formic acid, 4 l t^{-1} FM	NaB 0.30
E76	*L. plantarum* VTT E-78076, 1*10^6 cfu g^{-1} FM	NaB 0.30
E76+NaB	E76+Na-benzoate 0.3 g kg^{-1} FM	
BS	AIVBiostart, *L. plantarum* DSM 4409	NaB 0.30
BS+NaB	AIVBiostart+Na-benzoate 0.3 g kg^{-1} FM	

NaB = sodium benzoate, KS = potassium sorbate

with both FA0.4 and sodium benzoate, and consequently, in experiment II E76 and BS represent treatments E76 + NaB and BS + NaB.

The silos were covered with a plastic sheet, a plywood lid and weighed down using water containers, resulting in a force of 150 kg m^{-2}, and stored in an unheated barn. The silos were opened in three batches after 145 (±14) or 152 (±14) days of ensiling in experiments I and II, respectively. A layer approximately 15 cm from the top and bottom of silage was discarded, and representative samples were collected from the rest for the analysis of chemical composition, microbial quality and aerobic stability measurement.

Post-opening treatments and aerobic stability measurement

Immediately after opening the silos in experiment I, sodium benzoate, potassium sorbate, (Algol Oy, Finland, KS), or their combination (50:50) at three levels (0.15, 0.30, 0.45 g kg^{-1} FM) was applied and mixed well to UT, FA4 and LAB silages in water solutions (20 ml kg^{-1} FM). In experiment II, 0.30 g kg^{-1} FM sodium benzoate was added to UT, FA, E76 and BS silage.

For the aerobic stability measurement 350 g of each silage (intact and post opening treated) was inserted in an open plastic bag into a styrofoam box (volume 1.0 dm^3) in duplicates. On the top of the box was a hole (Ø 2 cm) for air to penetrate. A thermistor probe was inserted into the middle of the sample. The boxes were stored at room temperature (+21 ± 1°C). The temperature of the silages was recorded once a day for 10 days and aerobic stability data are presented as a cumulative difference (sample temperature minus ambient temperature).

Chemical and microbiological analysis

Chemical and microbial analysis of herbage and silage were carried out as described previously by Saarisalo et al. (2006). Dried samples were analysed for organic matter (OM), nitrogen (N), neutral detergent fibre (NDF) and for *in vitro* organic matter digestibility (OMD) by a modification of the method described by Nousiainen et al. (2003). Fresh samples were stored frozen (–20°C) until analysed for buffering capacity, WSC and soluble N. Oven determined silage DM was corrected with an equation given by Huida (1982). Water extract of silage was measured for pH, WSC, lactic acid, ethanol, ammonia-N and for VFAs (acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acid). Nitrogen and soluble N were measured from fresh samples using the Kjeldahl-method.

For quantitative microbial analysis an aseptically weighed 10 g sample of grass or silage was suspended in 90 ml of physiological saline containing 0.1% peptone and homogenized using a Stomacher (Seward 400, UK) for 30 s at medium power. The samples were analysed for counts of LAB (MRS agar, Oxoid; 30ºC, 3 d, anaerobic incubation), aerobic mesophilic bacteria (Plate Count Agar, Difco; 30ºC, 3 d), enterobacteria (Violet Red Bile Glucose (VRBG) Agar, Difco; 37ºC, 18–24 h), clostridia (SFP Agar Base, Difco; 37ºC, 2 d, anaerobic incubation), and yeasts and moulds (Yeast Glucose Chloramphenicol (YGC) Agar, Difco; 25ºC, 5 d). The microbiological analyses were carried out at the Technical Research Centre of Finland (VTT), Espoo.

Statistical analysis

The silage fermentation and microbial data were tested in both experiments using the SAS GLM procedure with the statistical model: $Y_i = \mu + T_i + e_i$ where Y_i is the observation, μ is the overall mean, T_i is the effect of treatment, and e_i is the residual error. The sums of squares for treatment effect were further separated by using orthogonal contrast into single degree of freedom comparisons. In experiment I the following comparisons were used: C1) UT vs. Additives, C2) FA4 vs. E76-treatments, C3) E76 alone vs. E76 + additives, C4) NaB vs. FA0.4, and C5) interaction C3 × C4. C4 and C5 were significant in a very few cases and are therefore only mentioned in the text. In experiment II,

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comparisons were: C1) UT vs. Additives, C2) FA vs. Inoculants, C3) E76 vs. BS, C4) Inoculants vs. Inoculants + NaB and C5) interaction C3 × C4. The two last ones were not significant for any of the parameters.

The effects of post-opening treatments on cumulative temperature in experiment I were tested separately for silages UT, FA4 and E76 and for each day using the following contrasts: C1) No vs. additive, C2) NaB vs. KS, C3) NaB or KS vs. NaB + KS, C4) Linear effect of NaB and KS, C5) Quadratic effect of NaB and KS, C6) interaction C2 × C4, C7) interaction C2 × C5, C8) interaction C3 × C4 and C9) interaction C3 × C5. The effects of post-opening treatments on cumulative temperature in experiment II were tested in two ways: Firstly the effect of silage treatment and post-opening addition of NaB using contrasts: C1) UT vs. additives, C2) FA vs. LABs, C3) E76 vs. BS, C4) effect of NaB, C5) interaction C1 × C4, C6) interaction C2 × C4 and C7) interaction C3 × C4.

Secondly the effect of time of NaB addition was tested with data from LAB silages: C1) E76 vs. BS, C2) effect of NaB, C3) time of NaB addition (into herbage and post-opening), C4) interaction C1 × C2 and C5) interaction C1 × C3.

Results

The herbages were wilted rapidly under optimal weather conditions so that relatively high DM contents (429 and 344 g kg^{-1} in experiments I and II, respectively, Table 2) were achieved within six hours. Herbage WSC was on average 134 and 117 g kg^{-1} DM in experiments I and II, respectively. In experiment I, there were less enterobacteria, LAB, clostridia and yeast in the FA4 treated herbage than in the UT and E76 treated herbage. In experiment II, there was a clear decrease in the

Table 2. Chemical composition (g kg^{-1} DM unless otherwise stated) and microbial quality (cfu g^{-1} FM) of the timothy and meadow fescue grass in experiments I and II. Means of three samples.

	Experiment I			Experiment II			
	UT	FA4	E76	UT	FA	E76	BS
Dry matter (g kg^{-1})	372	474	441	358	323	341	355
Ash	83.2	86.4	80.6	64.8	62.0	64.6	62.5
Neutral detergent fibre	529	498	520	584	566	588	588
Water soluble carbohydrates	128	142	131	112	135	114	108
Nitrogen	22.2	19.2	19.6	24.0	22.8	22.5	22.1
Soluble nitrogen (g kg^{-1} N)	274	252	256	378	285	331	374
Buffering capacity (mE kg^{-1}DM)	407	420	386	356	434	370	362
In vitro organic matter digestibility	791	804	795	763	771	749	760
Aerobic bacteria	7.8	7.6	7.9	6.8	5.6	6.4	6.5
Enterobacteria	5.5	4.5	4.8	3.8	<1.0	3.7	4.1
Lactic acid bacteria	4.6	2.5	6.5	5.5	3.3	5.7	5.8
Clostridia	1.2	0.5	2.5	1.2	<1.0	1.1	2.2
Yeasts	5.8	4.6	5.9	4.7	4.3	4.7	4.5
Moulds	5.8	5.5	6.0	5.1	2.0	5.2	5.1

UT = untreated, FA4 and FA = Formic acid, 4 l t^{-1}, E76 = *L. plantarum* VTT E-78076, BS = AIVBiostart, *L. plantarum* DSM 4409.

number of enterobacteria, LAB and moulds in the FA treated herbage compared to the other treatments.

Silage fermentation, microbiology and aerobic stability

Experiment I

In comparison with the additive treated silages UT silage had higher pH ($P < 0.001$) and contained more ash ($P < 0.05$) and less residual WSC ($P < 0.001$) (Table 3). The ethanol and acetic acid concentrations, and ammonia-N and soluble N were increased ($P < 0.001$) in the UT silage compared with the other treatments, while the opposite was observed for the lactic acid content ($P < 0.05$).

The FA4 treatment increased ash content ($P < 0.01$), pH and WSC (both $P < 0.001$) compared with the four E76 treatments. There was less lactic and acetic acid ($P < 0.001$) in the FA4 silage than in the E76 silages. The proportion of ammonia-N was increased in the FA4 silage compared with the E76 silages ($P < 0.001$) while soluble N was higher in the E76 than in the FA4 silage ($P < 0.05$).

Table 3. Chemical composition (g kg^{-1} DM unless otherwise stated), microbial quality (cfu g^{-1} FM) and aerobic stability of the silages in experiment I.

	Treatment							Statistical significance¤		
	UT	FA4	E76	E76+ NaB	E76+ FA0.4	E76+ FA0.4 +NaB	SEM	C1	C2	C3
Dry matter (g kg^{-1})	365	461	444	437	443	436	2.8			
Ash	89.3	89.3	85.0	86.1	86.5	86.5	1.00	*	**	
pH	4.54	4.78	4.06	4.07	4.06	4.05	0.034	***	***	
Water soluble carbohydrates	77	207	100	106	105	109	3.1	***	***	o
Ethanol	12.7	4.4	4.1	3.7	3.8	3.5	0.37	***		
Lactic acid	55.7	6.8	86.7	81.1	82.0	71.4	3.44	*	***	o
Acetic acid	10.6	5.9	7.2	7.2	7.0	7.2	0.15	***	***	
Volatile fatty acids	11.2	6.3	7.6	7.5	7.3	7.5	0.14	***	***	
Ammonia-N (g kg^{-1} N)	66.8	25.9	18.0	18.2	18.9	18.8	0.88	***	***	
Soluble N (g kg^{-1} N)	669	493	558	554	577	567	23.3	***	*	
Aerobic bacteria	7.8	7.0	5.4	5.4	5.5	5.7	0.33	***	**	
Enterobacteria	1.4	1.0	1.0	1.0	1.0	1.0	0.18	*		
Clostridia	1.2	1.0	1.2	1.1	1.2	1.0	0.14			
Lactic acid bacteria	7.7	7.0	5.8	5.7	5.4	5.6	0.32	***	**	
Moulds	2.4	2.6	2.1	2.1	1.7	1.7	0.53			
Yeasts	4.8	4.8	4.5	4.3	5.4	5.5	0.56			
Cumulative Temperature (°C)										
3 days	3.5	1.0	1.9	0.1	0.1	0.1	0.90	**		
5 days	16.9	8.7	10.2	2.1	3.3	1.5	3.43	**		o
7 days	32.6	27.6	26.5	7.5	16.2	4.7	5.17	*	*	**

UT = untreated, FA4 = Formic acid 4 l t^{-1}, E76 = *L. plantarum* VTT E-78076, FA0.4 = Formic acid 0.4 l t^{-1}, NaB = sodium benzoate 0.3 g kg^{-1} SEM = standard error of mean

¤Contrasts: C1) UT vs. additives, C2) FA4 vs. all E76, C3) E76 alone vs. E76+NaB and/or FA0.4. Only the significant contrasts are shown.

o = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$.

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Within the E76 silages the treatment with E76 alone tended ($P < 0.10$) to have less WSC (100 vs. 107 g kg^{-1} DM) and more lactic acid (86.7 vs. 78.2 g kg^{-1} DM) than the ones in combination with NaB and/or FA0.4. Within the E76 treated silages the E76 + FA0.4 + NaB treated silage contained the lowest lactic acid concentration (interaction $P < 0.05$).

In the UT silage the number of aerobic bacteria and LAB ($P < 0.001$) were increased compared with the treated silages. Also the enterobacteria count was slightly higher in the UT silage than in other silages ($P < 0.05$). The LAB and aerobic bacteria numbers were higher ($P < 0.01$) in the FA4 than in the E76 silages.

Aerobic instability expressed as a cumulative temperature was significantly increased in the UT silage compared to the other treatments after three, five ($P < 0.01$) and seven days ($P < 0.05$) exposure to air (Table 3 and Fig. 1a). The cumulative temperature of the FA4 treated silage was higher than in the E76 treated silages after seven days ($P < 0.05$). The cumulative temperature after five days tended to be higher in the E76 silage than in the silages treated with the combination of E76 and NaB and FA0.4 (difference 7.9°C, $P < 0.10$) and after seven days the difference was increased to 18°C ($P < 0.01$).

Five days after post-opening the number of aerobic bacteria and LAB were lower in additive treated silages as compared with UT ($P < 0.05$) (Fig. 2). After ten days UT silage had more aerobic bacteria ($P < 0.05$) and LAB ($P < 0.01$) and less moulds ($P < 0.05$) than additive treated silages. At the same time more aerobic bacteria ($P < 0.05$) and LAB ($P < 0.01$) were observed in FA than inoculated silages.

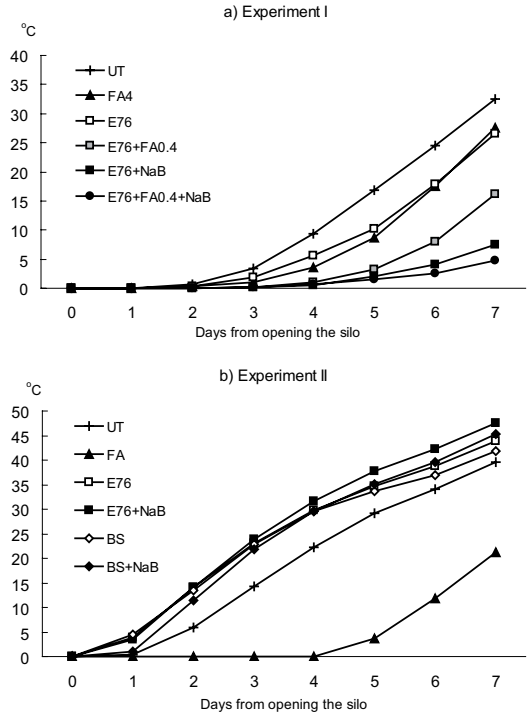

Fig. 1. Effect of additives on cumulative temperature of silages in experiments I (a) and II (b). UT = untreated, FA4 and FA = formic acid, E76 = *L. plantarum* VTT E-78076, BS = AIVBiostart, *L. plantarum* DSM 4409, NaB = sodium benzoate.

Experiment II

In experiment II, ash content tended to be ($P < 0.10$) higher in the UT silage than in the treated silages (Table 4). FA treatment restricted fermentation compared with the inoculated silages resulting in significantly higher pH, more residual WSC and less lactic acid (all $P < 0.001$). There was slightly less ethanol ($P < 0.10$) in the FA silage than in the inoculated silages. Ammonia-N was higher ($P < 0.001$) in the FA silage than in the inoculated while the opposite was observed for soluble N ($P < 0.01$). The only differences in the fermentation parameters between the E76 and BS silages were observed in the concentrations of acetic acid and VFA which were on average 2.17 and 2.60 g kg^{-1} DM higher ($P < 0.001$) in the BS than in the E76 silages.

The only significant differences in the microbial counts which were observed were in the comparison between FA and the inoculants. In the FA silage there were more enterobacteria ($P < 0.01$) and LAB and smaller yeasts count (both $P < 0.001$) than in the inoculated silages.

The cumulative temperature of the UT silage was lower than in the other treatments after three days (Table 4 and Fig. 1b). However, the most significant difference was improved stability of FA

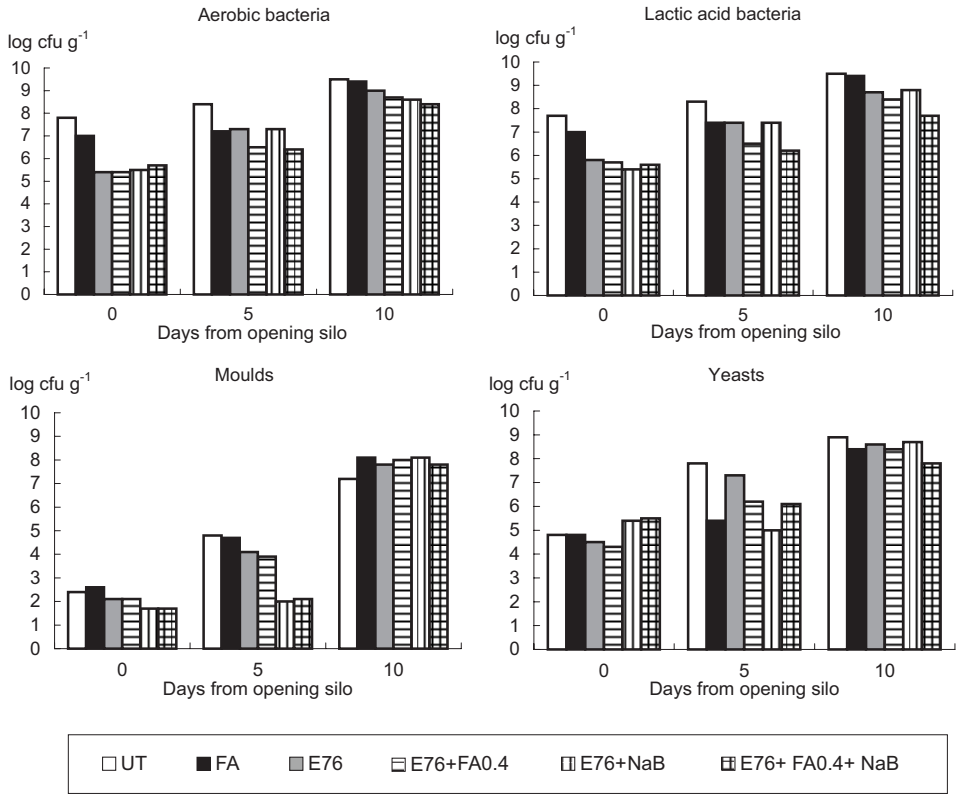

Fig. 2. Microbial quality of the silages at opening and after 5 and 10 days exposure to air in experiment I. UT = untreated, FA = formic acid, E76 = *L. plantarum* VTT E-78076, NaB = sodium benzoate.

silage compared to the inoculant silages at every time point ($P < 0.001$). There were no differences within the inoculated silages.

Effect of post-opening treatments on aerobic stability

In experiment I, sodium benzoate and potassium sorbate improved the aerobic stability of the UT silage from the second day after opening the silo ($P < 0.10$) and thereafter more clearly (Fig. 3a). The linear effect on NaB and KS was significant from day four ($P < 0.05$), and from the fifth day KS tended ($P < 0.10$) to be more effective than NaB. With FA silage NaB and KS only tended ($P < 0.10$) to improve aerobic stability during days three and four (Fig. 3b). With E76 silage (Fig. 3c) the effect of additives was significant from the second day (at least $P < 0.05$) and the linear effect of NaB and KS tended to be significant ($P < 0.10$) on day six and thereafter significant ($P < 0.05$). The rest of the contrasts were not significant. Changes in microbial quality during aerobic stability measurements are shown in Figure 2.

In experiment II, the addition of sodium benzoate post opening improved the aerobic stability of the UT, E76 and BS silages by delaying the onset of warming by one day (Fig. 4). Sodium benzoate did not improve aerobic stability of the FA silage. There was a statistically significant difference in the effect of NaB between the inoculants and FA during the first four days (at least $P < 0.05$,

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Table 4. Chemical composition (g kg^{-1} DM unless otherwise stated), microbial quality (cfu g^{-1} FM) and aerobic stability of the silages in experiment II.

	Treatment							Statistical significance¤		
	UT	FA	E76	E76+ NaB	BS	BS+ NaB	SEM	C1	C2	C3
Dry matter, g kg^{-1}	347	313	329	329	344	343	2.7	***	***	***
Ash	67.5	65.7	66.0	66.3	65.1	66.2	0.71	o		
pH	4.10	4.34	3.92	3.92	3.92	3.93	0.034	*	***	
Water soluble carbohydrates	40	138	36	42	41	44	2.6	***	***	
Ethanol	9.7	7.9	10.5	10.2	8.2	8.0	0.67		o	
Lactic acid	86	11	101	99	100	97	1.6	*	***	
Acetic acid	9.7	8.0	6.3	7.2	8.9	9.0	0.47	**		***
Volatile fatty acids	10.0	8.3	6.5	7.4	9.2	9.9	0.60	*		***
Ammonia-N, g kg^{-1} N	51.9	28.4	22.7	22.6	22.9	23.1	1.14	***	***	
Soluble N, g kg^{-1} N	683	590	648	633	655	650	14.9	*	**	
Neutral detergent fibre	571	566	573	573	574	574	2.9		*	
Aerobic bacteria	6.7	5.9	6.2	6.5	6.4	6.2	0.22	o		
Enterobacteria	0.5	1.7	0.5	0.5	0.5	0.5	0.27		**	
Clostridia	0.5	1.0	0.5	0.5	0.5	1.0	0.15		*	
Lactic acid bacteria	6.0	7.2	6.0	6.2	6.1	6.0	0.22		***	
Moulds	1.4	1.7	1.1	1.0	1.2	1.7	0.24			
Yeasts	5.9	4.2	6.1	6.4	6.1	6.0	0.32		***	
Cumulative Temperature °C										
3 days	14.3	0.0	23.1	23.9	22.8	21.9	1.70	*	***	
5 days	29.2	3.7	34.7	37.7	33.7	35.2	2.65		***	
7 days	39.6	21.2	43.8	47.6	41.9	45.3	3.39		***	

UT = untreated, FA = Formic acid 4 l t^{-1}, E76 = *L. plantarum* VTT E-78076, BS = AIVBiostart, NaB = sodium benzoate 0.3 g kg^{-1} FM, SEM = Standard error of mean

¤ Contrasts: C1) UT vs. Additives; C2) FA vs. Inoculats; C3) E76 vs. BS. Only the significant contrasts are shown.
o = P < 0.10, * = P < 0.05, ** = P < 0.01, *** = P < 0.001

and interaction P < 0.10). With the inoculated silages the post opening addition of NaB tended to be more effective in improving the stability than NaB addition during the silo filling (time of addition P < 0.05). The rest of the contrasts were not significant.

Discussion

The compositions of the herbages in experiments I and II were good in terms of ensilability with sufficient WSC content (on average 57 and 40 g kg^{-1} FM in experiments I and II, respectively) and a typical buffering capacity for the grass species used. The lower DM content in the UT herbage in experiment I was unexpected and was probably caused by varying yield and DM concentration of grass on the field.

Formic acid 4 l t^{-1} and the inoculants were applied during harvesting resulting in immediate pH drop on herbage as in typical ensiling. NaB and FA0.4 were applied during filling the silos. These additives are expected to have minor effects on composition of grass between harvesting and ensiling. The WSC content in the herbage, sampled

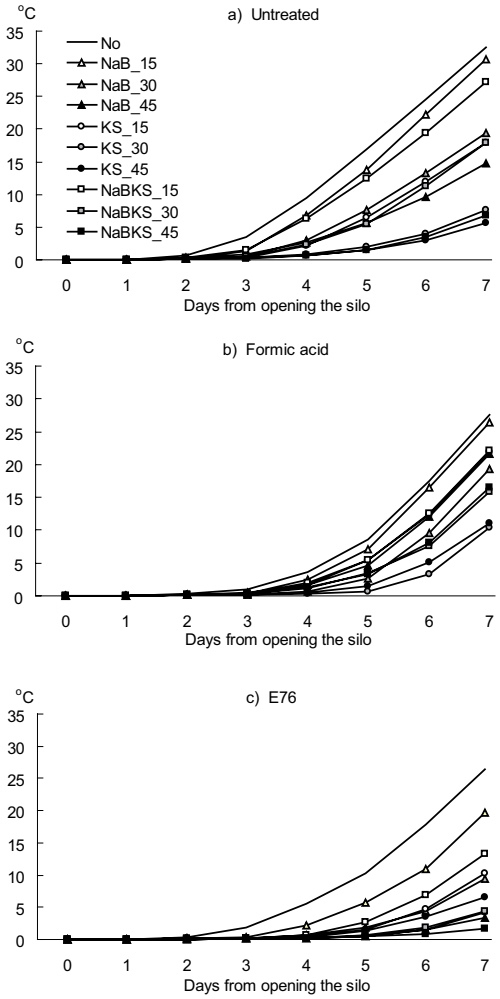

Fig. 3. Effects of post-opening treatments with sodium benzoate (NaB) and potassium sorbate (KS) at three application rates (0.15, 0.30 and 0.45 g kg^{-1} FM) on cumulative temperature of untreated (a), formic acid treated (b) and inoculant (E76) treated (c) silages in experiment I.

during filling the silos, 2–3 hours after additive application, was higher in the FA treated than in the untreated or inoculated herbage agreeing with the observations of Keady and Murphy (1997). Respectively, the NDF content was smaller in the FA herbage suggesting WSC releasing acid hydrolysis. Another possible reason for the highest WSC

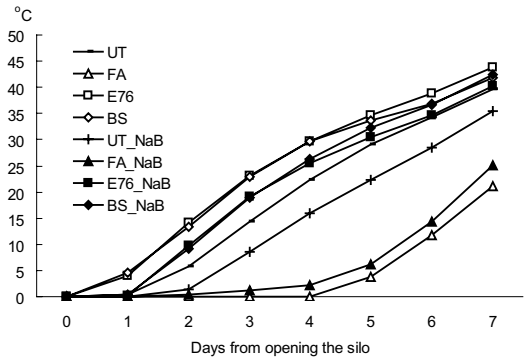

Fig. 4. Effect of post opening addition of sodium benzoate (NaB) on aerobic stability of silages in experiment II. UT = untreated, FA = formic acid, E76 = *L. plantarum* VTT E-78076, BS = AIVBiostart, *L. plantarum* DSM 4409

content in the FA herbage is inhibition of respiration between the time of additive application and freezing of the samples.

Effect of treatments on silage fermentation

It is well documented that the fermentation quality of low DM grass silage can be improved by FA based silage additives (Waldo 1977, Jaakkola et al. 1991, Kung et al. 2003, Jaakkola et al. 2006) while with inoculants results have been more variable (Weinberg and Muck 1996, Pobednov et al. 1997, Kung et al. 2003). With the low DM grass silages the positive effects of inoculants on silage fermentation depend on the adequate amount of WSC (Anderson et al. 1989) and often no effect compared to untreated has been observed with low initial WSC (e.g. Keady and Murphy 1997, Yan et al. 1998). Fewer studies have been conducted to compare additives with rapid wilted, high DM (≥330 g kg^{-1}) grass silages.

In the present experiments, all the silages were of good quality and none contained excessive concentrations of acetic, butyric or other VFAs, or

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ethanol. However, all the additives improved the fermentation quality compared with the UT silage, especially in terms of the ammonia-N, which was 45 and 28 g kg^{-1} N higher in the UT than in the additive treated silages in experiments I and II, respectively. In experiment I, the difference in the fermentation quality of the UT and the additive treated silages was greater than in experiment II. This was probably caused by the lower DM content in the UT than in other silages in experiment I, resulting in more extensive fermentation of the UT silage.

Despite the reasonable high DM content of silage in both the experiments, there were noticeable differences in the fermentation type between the FA and inoculant treatments, though both were equally effective in decreasing ammonia production. All the treatments resulted in high concentrations of residual WSC, which resulted mainly from the moderate formation of fermentation acids. In the FA silages, formic acid, together with the high DM, restricted production of lactic and other fermentation acids, resulting in a pH 0.7 and 0.4 higher than in the inoculated silages in experiments I and II, respectively. The LAB inoculants enhanced lactic acid production, resulting in lower pH, which improves hygienic quality regarding potential silage transmitted pathogens like *Listeria*, coliforms, *Bacillus* and *Clostridia* (Jonsson et al. 1990, McDonald et al. 1991). The capability of inoculants to improve silage fermentation at high dry matter was also observed by Jonsson et al. (1990) and Driehuis et al. (1997). Regarding the novel LAB inoculant, the results agree with the previous laboratory scale experiment in which E76 resulted in fast lactic acid production, a drop in pH and restricted ammonia production (Saarisalo et al. 2006). E76 was equally effective as the commercial inoculant strain in BS (*L. plantarum* DSM 4409).

Effect of treatments on aerobic stability

Aerobic deterioration decreases the nutritive value of silage, impairs the hygienic quality and, in addition to animals, may cause health risks to people working with animals and even to consumers via milk as reviewed by Woolford (1990), Lindgren (1991) and Wilkinson (1999). The majority of research on the factors affecting aerobic stability has focused on maize and small grain cereal silages (Driehuis et al. 1999, Weinberg et al. 1999, Uriarte and Bolsen 2001, Danner et al. 2003) indicating that the aerobic instability has been a greater problem with those materials having a higher DM content and a coarser physical structure than grass. It is possible that the fermentation pattern, microbial flora and course of aerobic deterioration in maize and small grain cereal silages are somewhat different from wilted grass silage. In both of our experiments, aerobic stability of grass silages was relatively poor, since increased temperature was observed already from day one (experiment I) or day three (experiment II) after opening the silos. The complexity of the factors affecting aerobic stability of silage was highlighted and some of the results disagree with the previous observations on the relationship between the chemical composition and the aerobic stability.

A high DM content has been mentioned as a factor impairing the stability of grass silage (Yan et al. 1998, Wyss 1999). However, the UT silage had the lowest stability in experiment I despite having the lowest DM content. In addition, the silages in experiment I with an average DM of 431 g kg^{-1} were more stable than the silages in experiment II with an average DM of 327 g kg^{-1}. These observations suggest that DM content of silage alone does not predict the susceptibility to aerobic deterioration well.

Another major factor considered to affect aerobic stability is the fermentation type of silage. In particular, extensive heterolactic fermentation and butyric and acetic acid have improved stability (McDonald et al. 1991, Danner et al. 2003). In our experiments, the UT silage with the highest acetic acid concentration was less stable than the FA treated restrictively fermented silage. Furthermore, the inconsistency between the fermentation type and aerobic stability was emphasised by the results with the LAB inoculants. Despite similar homolactic fermentation in experiments I and II, the inoculants produced dissimilar effects on aerobic

stability. In experiment I, E76 improved aerobic stability compared to UT, while in experiment II, both inoculants produced less stable silage than UT and especially FA. Variable effects of homolactic inoculants are not rare in the literature (McDonald et al. 1991, Uriarte and Bolsen 2001).

The application rate of 4 l FA t^{-1} grass, as recommended in Finland, resulted in good aerobic stability, especially in experiment II, despite restricted fermentation, high WSC content and high pH, which have been considered as risk factors for aerobic stability. Another noteworthy observation was that the yeast count was not increased in the FA silages as often observed (McDonald et al. 1991, Uriarte and Bolsen 2001). The varying effect of FA on aerobic stability is not uncommon and possibly due to different microorganisms responsible for aerobic deterioration (Kung et al. 2003). An explanation for our good results with rather high rate of FA might be the effective consolidation of the acid treated herbage during silo filling, leading to increased storage density and thereby decreased air infiltration into the silage.

A way to decrease both aerobic instability and improve silage fermentation is to combine homolactic LAB inoculant with chemical additives. Restriction of fermentation by wilting and LAB inoculation with chemical preservatives can be regarded as an application of the hurdle technology (Leistner and Gorris 1995). For example, combination of an inoculant and sodium formate was studied by Weissbach et al. (1991). Sodium benzoate and potassium sorbate are weak-acid preservatives effective against yeasts and moulds (Woolford 1975) and commonly used as food preservatives. Their potential as antimicrobial agent depends on the proportion of undissociated acids which pass through the cell membranes and liberate protons, thus acidifying the cytoplasm and preventing growth of microbes (Lambert and Stratford 1999). In addition, the effect depends on pH as the proportion of the undissociated acids increases with declining pH. In the present experiments, two different methods to add chemical agents were used, either application during ensiling or post-opening the silos. Using the latter method it is possible to study the potential of these additives with more treatments than it would have been practically possible within an ensiling experiment. A similar approach has also been used by Weinberg et al. (1993) in a study on effects of acetic and propionic acid.

The chemical additives FA0.4 (0.4 l t^{-1} FM) in experiment I, and NaB (0.3 g kg^{-1} FM) in both experiments applied to the inoculated silages during ensiling did not have any adverse effect on the efficiency of inoculants since silage fermentation was not affected. However, FA0.4 applied as free acid had to be added separately since in a previous laboratory experiment LAB was eliminated when applied mixed with the same amount of FA as in the current experiment.

In experiment I, FA0.4 and/or NaB improved aerobic stability of the E76 silage even though the yeast count was smaller in E76 without the chemical additives than in E76 + FA04 and E76 + FA0.4 + NaB silages. There were no other clear differences in the microbial counts that could explain this improved aerobic stability of inoculated silages. On the other hand, microbial changes during exposure to air (Fig. 2) clearly reflected aerobic stability as the number of moulds and yeasts did not increase in the E76 + chemical additive silages in which the smallest temperature increase was observed after five days. This supports the hypothesis that the chemical additives in combination with an inoculant restrict microbes responsible for aerobic deterioration, which is in line with the results of Weissbach et al. (1991) and Rammer et al. (1999). In experiment II, NaB added at the same rate as in experiment I had only a minor effect on aerobic stability. Rammer et al. (1999) reported prolonged storage stability with 0.20 and 0.40 g kg^{-1} NaB in combination with an inoculant applied to grass and grass-legume herbage. However, fermentation parameters were not reported. According to Lingvall and Lättemäe (1999), 0.8 g kg^{-1} NaB or 0.69 NaB + 0.21 sodium propionate was enough to control aerobic stability of baled grass silage (DM 330–350 g kg^{-1}, pH 4.3–4.4). Kleinschmit et al. (2005) found that 1 g kg^{-1} of NaB and also 1 g kg^{-1} maize silage of KS plus EDTA improved stability. Regarding the variability in reasons for onset of deterioration, it is clear that the minimum effective ap-

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plication rate also varies. Sodium benzoate and potassium sorbate applied post-opening were more effective in improving the stability of inoculated silages than FA silages, which can be explained by the clear difference in silage pH affecting the proportion of dissociated acids.

Within the experiments the relationship between the microbial quality and aerobic stability was not straightforward. However, the poorer aerobic stability of the inoculated silages in experiment II could be explained by the yeast count which was a hundred times higher (log 4 vs. log 6) in experiment II than in experiment I. The number of aerobic bacteria was also ten times higher in experiment II. In the present study, microbial population was examined quantitatively, only at a general level. More detailed characterisation of the yeast population would be needed for explaining the role of lactate fermenting yeasts in the aerobic spoilage process. Yeast population differences in first and second cut silages may also play an important role.

Antimicrobial properties of E76 might be a factor in ensuring rapid proliferation at early phases of silage fermentation and consequently a good fermentation pattern. However, as observed in the previous study (Saarisalo et al. 2006), LAB numbers in final silages were smaller in inoculated than in the restrictively fermented or in UT silage, probably due to low pH and autolysis.

In conclusion, the novel LAB inoculant was equally effective in improving fermentation quality, and especially in reducing ammonia formation in grass silage, compared with the commercial LAB inoculant. However, the aerobic stability of inoculant-treated silages was poorer than that of formic acid treated or untreated silages in one of the experiments. The results suggest that the antimicrobial properties of E76 were not effective enough to improve aerobic instability. One option to overcome this problem is to use chemical additives in combination with the inoculants. The results suggest that the minimum effective application rate of sodium benzoate varies.

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SELOSTUS

Maitohappobakteerien, muurahaishapon, natriumbentsoaatin ja kaliumsorbaatin vaikutus esikuivatun säilörehun käymislaatuun ja aerobiseen stabiilisuuteen

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Maa- ja elintarviketalouden tutkimuskeskus ja VTT

Esikuivatus vähentää säilörehun käymisen laatuongelmia, mutta voi johtaa siihen, että rehut lämpenevät herkemmin siilon avaamisen jälkeen. Kahdessa kokeessa tutkittiin uuden maitohappobakteerikannan, *Lactobacillus plantarum* VTT E-78076 (E76), tehokkuutta säilörehun biologisena säilöntävalmisteena. Säilörehujen käymislaadun lisäksi tutkittiin rehujen jälkipilaantumisherkkyyttä eli aerobista stabiilisuutta ja mahdollisuutta parantaa sitä yhdistämällä biologinen ja kemiallinen säilöntävalmiste.

Säilörehujen raaka-aine oli esikuivattua timotei-nurminataa, jonka kuiva-ainepitoisuus oli ensimmäisessä kokeessa 429 g/kg ja toisessa 344 g/kg. Säilöntäainekäsittelyjä oli kuusi. Kokeen I käsittelyt olivat E76 yksin (10^6 pmy/g), E76 yhdessä natriumbentsoaatin (0,30 g/kg ruohoa) tai lievän muurahaishapon (0,40 g/kg ruohoa) sekä näiden yhdistelmän kanssa. Kokeen II käsittelyt olivat E76 ja kaupallinen biologinen valmiste (AIVBioprofit, *L. plantarum* DSM 4409, 10^6 pmy/g) sekä yksin että yhdessä natriumbentsoaatin (0,30 g/kg ruohoa) kanssa. Kontrolleina molemmissa kokeissa olivat käsittelemätön eli painorehu ja muurahaishappopohjainen säilöntäaine (AIV2Plus 5 l tonnille). Aerobisen stabiilisuuden parantamista tutkittiin myös lisäämällä natriumbentsoaattia ja kaliumsorbaattia rehuihin siilojen avaamisen jälkeen.

Uusi maitohappobakteerikanta tuotti painorehua paremman ja kaupalliseen valmisteeseen verrattuna yhtä hyvän käymislaadun. Maitohappobakteerirehujen aerobinen stabiilisuus oli kuitenkin molemmissa kokeissa huonompi kuin muurahaishapporehun, ja toisessa kokeessa myös painorehu oli stabiilimpaa. Aerobista stabiilisuutta voitiin parantaa yhdistämällä maitohappobakteeri ja kemiallinen säilöntäaine.