A new probiotic and bacteriocin-producing strain of *Enterococcus faecium* EF9296 and its use in grass ensiling

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ABSTRACT: A new probiotic, bacteriocin-producing strain of *Enterococcus faecium* EF9296 (isolate of our laboratory) was used in grass ensiling as an inoculant. The antimicrobial effect as well as the nutritive quality of silage was evaluated along with the aerobic stability test. Grass was ensiled with EF9296 strain (10^9 colony-forming units per gram of fresh material) and the silage without EF9296 was also prepared. The silages were processed in 1-litre plastic bottles. The samples were collected at days 0–1 (start of experiment), 7, 14 and 105 of ensiling to check the microbial status and chemical parameters of silage. At the end of ensiling period (day 105) the silages were subjected to an aerobic stability test for 7 days. The EF9296 strain survived and colonized the silage in sufficient counts (10^9–10^{10} cfu/g). The total counts of enterococci and lactic acid bacteria were increased. In the silage inoculated with EF9296 strain, a reduction in *E. coli*, enterobacteriae, staphylococci and *Bacillus*-like bacteria was noted mainly at day 7 and 14 of ensiling. Moreover, the counts of *Listeria* and moulds were reduced during the aerobic stability test. The silage inoculation resulted in a more rapid drop of pH, higher level of lactic acid and in a decrease in the concentration of acetic and butyric acid. The effect of *E. faecium* EF9296 on the value of pH was prolonged until the end of the experiment (including the aerobic stability test, below 4.3). During the whole ensiling period an increase in DM content in the experimental silage was detected. The *E. faecium* EF9296 strain represents a promising silage additive to produce silage of good quality and/or to prevent silage contamination.

Keywords: probiotic; bacteriocin; ensiling; *Enterococcus faecium*; grass

Silage production is a method of moist forage preservation which is widely used all over the world, accounting for more than 287 million tons of dry matter (DM) stored annually in the EU (Saele, 2002). It is based on natural fermentation when lactic acid bacteria (LAB) ferment water soluble carbohydrates to organic acids, mainly lactic acid, under anaerobic conditions. As a result, the pH decreases, inhibiting detrimental anaerobes, and so the moist forage is preserved (Merry and Davies, 1999). In order to improve the ensiling process, various chemical and biological additives have been developed. The biological additives are advantageous because they are safe and easy to use, non-corrosive to machinery, do not pollute the environment and are regarded as natural products (Filya et al., 2000). Silage inoculants containing principally LAB have become the dominant additives in many parts of the world not only for their convenience and safety but also because they are expected to control microbial

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events during silage fermentation (Weinberg and Muck, 1996). Although the effects of inoculants on the preservation and nutritive value of silage have often been inconsistent, positive outcomes such as higher lactate:acetate ratios, lower ammonia N, decreased DM losses, increased digestibility, improved aerobic stability and enhanced growth performance have been reported (Henderson, 1993; McAllister et al., 1995, 1998; Zahiroddini et al., 2004). Most available inoculants consist of selected strains of homofermentative LAB, such as *Lactobacillus plantarum*, *Pediococcus* sp. and *Enterococcus faecium*, which are of different origin (Weinberg and Muck, 1996; Přikryl, 2006). The inoculation rates of these products are usually 10^5–10^6 viable cells/g, which is often sufficient for the inoculant LAB to outgrow the epiphytic LAB and become the predominant population in silage. Moreover, the ability of LAB to produce natural antimicrobial substances – organic acids, diacetyl, hydrogen peroxide and bacteriocins is also well known (Klaenhammer, 1993).

Enterococcus faecium EF9296 represents a new probiotic and bacteriocin-producing strain, isolated from silage, the properties of which were reported in our previous study (Marciňáková et al., 2004). The EF9296 strain possesses a sufficient adhesive capability to mucus (4.7–5.5 log 10 bacteria adhered per microtitre plate well), it is a bile resistant, lactic acid producing, ureolytic strain, the bacteriocin substance of which acts especially against listeriae.

Many factors affect the aerobic stability of silages. Oxygen is detrimental to silage quality because it enables aerobic spoilage microorganisms such as yeasts and moulds to grow (Woolford, 1990). Silage may be exposed to air during storage and unloading for feeding, and so it is susceptible to spoilage, especially in warm climates (Ashbell et al., 2002). During the silo unloading, silage is normally fully exposed to air, which could result in an increase in temperature and aerobic deterioration. The stability of silage in the presence of oxygen is a very important factor determining its subsequent nutritional quality and feeding value (Filya, 2004).

The aim of the present study was to test the antimicrobial effect of a new probiotic and bacteriocin-producing strain of *Enterococcus faecium* EF9296 – isolate from silage after its inoculation in silage and to check the ensiling process, nutritive value and aerobic stability of silage in a model experiment to recommend it for its further use as silage additive.

MATERIAL AND METHODS

Preparation of silage inoculant

Enterococcus faecium EF9296, silage isolate was cultivated in Todd-Hewitt broth (Becton and Dickinson, Cockeysville, USA). To distinguish the EF9296 strain from the other enterococci, a strain marked by rifampicin was used. It was obtained by subsequent cultivation of the strain using Todd-Hewitt agar (Becton and Dickinson) with rifampicin (100 μg/ml) at 37°C.

Schedule of ensiling experiment

Grass (*Elytrigia repens*) in mid-bloom was harvested and left to wilt for 24 h. The wilted forage was chopped to particles of about 4–6 cm in size. The chopped forage was mixed and divided into equal portions (10 kg) for the application of 2 treatments. The control forage silage was without EF9296 strain. The experimental forage was inoculated by spraying with *E. faecium* EF9296 (10^9 cfu/ml). Forage from each treatment was ensiled in 1-litre plastic bottles equipped with a lid that enabled the gas release only. Before filling, each bottle was weighed and then filled with chopped forage to have almost 1 kg. There were 10 bottles per treatment stored at an ambient temperature (21°C) and continually checked. Two bottles from each treatment were sampled and chemical and microbial analyses were done at day 0–1 (start of the experiment), 7, 14 and 105 of ensiling in four parallels.

Microbial and chemical analyses

To enumerate bacteria, 10 g of silage was mixed with 90 ml of Ringer solution (pH 7.0, Basingstoke, England); 100 μl aliquots of serial dilutions (10^{-1} to 10^{-9}) were plated on the following media: Todd-Hewitt agar with rifampicin to enumerate EF9296 strain, M-*Enterococcus* agar for enterococci, mannitol salt agar (Becton and Dickinson) for staphylococci, De Man-Rogosa-Sharpe agar (MRS, Merck, Darmstadt, Germany) to detect LAB, Mc Bride *Listeria* agar and Oxford agar to isolate listeriae, Mac Conkey agar (Becton and Dickinson) for *E. coli* and other *Enterobacteriae*, *Bacillus cereus* agar (Biomark, India) for *Bacillus*-like bacteria and chloramphenicol yeast glucose agar (Biomark) for

Table 1a. Bacteria in silage (log cfu/g) inoculated with *Enterococcus faecium* EF9296 strain and in control silage (without inoculant)

Ensiling	EF9296 strain	Enterococci	LAB	*E. coli*
Day 0				
Control silage	ND	3.97 (0.67)	3.78 (0.78)	ND
EF9296 silage	4.75 (0.01)	4.92 (0.06)	4.78 (0.04)	0.65 (0.65)
Day 7				
Control silage	ND	6.68 (0.08)	6.43 (0.13)	4.79 (0.10)
EF9296 silage	7.95 (0.01)***	8.60 (0.01)***	6.56 (0.03)	2.97 (1.13)
Day 14				
Control silage	ND	9.70 (0.36)	8.04 (0.09)	5.24 (0.07)
EF9296 silage	10.16 (0.39)*	9.63 (0.29)	7.45 (0.15)	4.16 (0.16)*
Day 105				
Control silage	ND	10.29 (0.06)	7.52 (0.52)	4.98 (0.91)
EF9296 silage	9.15 (0.15)	9.85 (0.55)	7.84 (0.15)	4.23 (0.23)

LAB-lactic acid bacteria; *$P < 0.05$; ***$P < 0.001$ significant differences; the values of SD (standard deviations) are in brackets; ND = not determined

yeasts and moulds. Enterococci, *E. coli*, enterobacteriae and *Bacillus*-like bacteria were cultivated at 37°C for 24–48 hours. LAB and staphylococci were cultivated at 30°C for 48 hours and *Listeria*-like bacteria at 30°C for 48–72 hours. Yeasts and moulds were cultivated at room temperature for 3 days. Numbers of colony-forming units (cfu) were expressed in log 10 cfu/ml/g ± SD.

Chemical analyses were performed in four parallels. The dry matter (DM) content of silages was determined by drying at 105°C for 24 hours in a fan-assisted oven (Declaration of the Ministry of

Table 1b. Bacteria in silage (log cfu/g) inoculated with *Enterococcus faecium* EF9296 strain and in control silage (without inoculant)

Ensiling	Enterobacteria	Staphylo-coccus*	*Listeriae*	*Bacillus*-like	Yeasts	Moulds
Day 0–1						
Control silage	1.38 (1.38)	3.38 (0.08)	4.14 (0.24)	4.80 (0.08)	4.17 (0.13)	4.33 (0.10)
EF9296 silage	3.00 (1.00)	3.57 (0.27)	4.47 (0.24)	4.94 (0.12)	2.23 (0.54)	4.48 (0.41)
Day 7						
Control silage	7.03 (0.43)	4.01 (0.06)	ND	7.46 (0.12)	1.53 (0.53)	1.58 (0.58)
EF9296 silage	4.46 (0.86)	3.69 (1.09)	ND	5.18 (1.11)	0.92 (0.92)	1.86 (0.17)
Day 14						
Control silage	6.01 (0.06)	5.41 (0.21)	ND	7.47 (0.13)	0.95 (0.95)	3.03 (0.03)
EF9296 silage	5.52 (0.48)	4.08 (0.31)*	ND	4.73 (0.73)*	3.53 (0.23)	3.42 (0.42)
Day 105						
Control silage	4.30 (1.00)	5.23 (0.23)	6.45 (0.55)	3.73 (3.73)	4.19 (0.72)	2.44 (0.19)
EF9296 silage	4.58 (1.58)	4.65 (0.35)	4.15 (0.25)*	4.00 (4.00)	2.31 (2.31)	3.67 (0.47)

*$P < 0.05$ significant differences from the control; the values of SD (standard deviations) are in brackets; ND = not determined

Agriculture of the Slovak Republic, 1998). Crude protein (CP) was determined by the Kjeldahl method (Declaration of the Ministry of Agriculture of the Slovak Republic, 1998). To measure pH, the water extract of 100 g of silages and the equipment 3310 (Jenway, Essex, United Kingdom) were used. The amount of lactic acid (LA), acetic acid (AA) and butyric acid (BA) was measured by capillary isotachophoresis using a ZKI 01 analyzer (Slovak Republic).

Aerobic stability test

At the end of the 105-day ensiling period, the silages were subjected to the aerobic stability test lasting 7 days in a bottle system (Ashbell et al., 1991). At day 0–1 (start of the aerobic stability test), at days 2, 5 and 7, two bottles of each treatment were emptied to measure pH, lactic, acetic and butyric acid and to enumerate EF9296 strain. Moreover, the counts of *Listeria* sp., yeasts and moulds were determined by the method mentioned above.

Statistical evaluation of the results was performed by Student's *t*-test (SAS, 1995) with the level of significance set at $P < 0.05$.

RESULTS

E. faecium EF9296 counts were increased from the initial value 4.75 ± 0.01 log cfu/g at day 0–1 to 7.95 ± 0.00 log cfu/g ($P < 0.001$) at day 7 (Table 1a). At day 14, EF9296 strain reached 10.16 ± 0.39 log cfu/g ($P < 0.05$) and at the end of ensiling (at day 105) its count was 9.15 ± 0.15 log cfu/g. The counts of total enterococci in the silage with EF9296 strain increased during the ensiling period from 4.92 ± 0.06 log cfu/g (at day 0–1) to 9.85 ± 0.55 cfu/g (at day 105, Table 1a). A significant increase in enterococci was found in the experimental silage after 7 days of ensiling compared with the control silage (difference of 1.92 log cycle, $P < 0.001$). The counts of LAB in silage with EF9296 strain increased from 4.78 ± 0.04 cfu/g at the start of ensiling to 7.84 ± 0.15 cfu/g at day 105. The counts of *E. coli* were decreased in silage inoculated with EF9296 strain (at day 7 a difference of 1.82 log was found, at day 14 a difference of 1.08 log, $P < 0.05$, Table 1a) although they were not eliminated. After the silage inoculation with EF9296 strain, a decrease in enterobacteriae was found at day 7 (difference

Table 2. Chemical analyses of silage inoculated with *Enterococcus faecium* EF9296 strain and of control silage (without inoculant) during ensiling

Ensiling	pH	Dry matter (%)	Nitrogen (mg %)	N-cont. comp. (mg %)	Lactic acid (g/kg)	Acetic acid (g/kg)	Butyric acid (g/kg)
Day 0–1							
Control silage	5.72	27.87 (1.28)	2.17 (0.07)	13.55 (0.50)	1.19 (0.11)	1.76 (0.66)	ND
EF9296 silage	6.06	28.63 (0.03)	2.05 (0.05)	12.80 (0.30)	8.38 (0.74)	1.62 (0.22)	ND
Day 7							
Control silage	4.97 (0.84)	27.03 (0.17)	2.27 (0.02)	14.18 (0.12)	3.79 (0.47)	4.50 (0.01)	0.11 (0.11)
EF9296 silage	5.04 (0.89)	28.41 (0.94)*	2.47 (0.02)	15.45 (0.15)	9.73 (4.50)	2.42 (0.58)*	0.00 (0.00)
Day 14							
Control silage	6.39 (0.30)	26.28 (0.22)	2.65 (0.15)	16.55 (0.95)	2.25 (0.35)	3.92 (0.08)	0.22 (0.00)
EF9296 silage	5.13 (0.04)*	28.60 (0.26)***	2.37 (0.17)	14.82 (1.07)	12.45 (1.06)**	2.00 (0.16) **	0.33 (0.33)
Day 105							
Control silage	5.26 (0.07)	25.18 (1.12)	2.03 (0.04)	12.71 (0.24)	7.03 (1.19)	4.30 (1.19)	1.34 (0.12)
EF9296 silage	4.28 (0.06)**	26.30 (0.66)	2.30 (0.12)	14.37 (0.77)	21.23 (0.65)**	3.57 (0.64)	0.85 (0.12)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ significant differences from the controls; the values of SD (standard deviations) are in brackets; ND = not detected

of 2.57 log) and at day 14 (difference of 0.49 log, Table 1b) compared with the control silage. The highest decrease in staphylococci was noted at day 14 in the silage with EF9296 strain (difference of 1.33 log, $P < 0.05$) in comparison with the control silage. *Bacillus*-like colonies were reduced at day 14 (decrease of 2.74 log, $P < 0.05$, Table 1b) in EF9296 silage compared with the control silage. The cells of *Listeria* sp. were decreased at the end of ensiling ($P < 0.05$, Table 1b). In addition, at the end of experiment (day 105) a drop of the yeast population was determined in the experimental silage (difference of 1.88 log in EF9296 silage) compared with the control silage.

The silage inoculated with *E. faecium* EF9296 strain appeared to be of good quality, exhibiting low pH (below 4.6; the pH decrease in the experimental silage was significant, $P < 0.05$, a difference of 1.26 log after 14 days of ensiling), high concentrations of lactic acid and low concentrations of acetic and butyric acid (Table 2). A significant increase in LA was observed at day 14 between the control and experimental silage (difference of 10.20 g/kg, $P < 0.01$). Moreover, in the experimental silage a significant increase in LA was detected at day 105 (difference of 14, 20 g/kg, $P < 0.01$) compared to the control silage. The lowest levels of AA were found at day 7 (difference of 2.08 g/kg, $P < 0.05$) and at day 14 of ensiling (difference of 1.93 g/kg, $P < 0.01$) compared with the control silage. In the silage with EF9296 strain a significant increase in DM content was observed at day 7 ($P < 0.05$) as well as at day 14 ($P < 0.001$) of ensiling compared to the control silage. No influences of the inoculant on the values of nitrogen as well as on the values of nitrogen-containing compounds (Table 2) were detected.

In the aerobic stability test, a decrease in the EF9296 strain was noted (from 9.15 ± 0.15 log cfu/g to 4.16 ± 0.16 log cfu/g after 7 days, Table 3a). Concerning the counts of *listeriae*, a reduction was detected in EF9296 silage (difference of 2.30 log, $P < 0.05$; 2 days after exposure to air a difference of 2.2 log) compared to the control silage. Although the lower counts of yeasts were determined in EF9296 silage at the end of ensiling (Table 1b), during the aerobic stability test an increase in the yeast population was noted in EF9296 silage in comparison with the control silage. On the other hand, the mould population decreased during the exposure to air (at 7 day) in EF9296 silage compared with the control silage (Table 3a). Moreover, in EF9296 silage a significant decline of pH was found at day 0–1 of the aerobic stability testing (difference of 0.98 log, $P < 0.01$) with prolonging the status until the end of the test (during the exposure to air, below 4.3). High concentrations of LA and low concentrations of acetic and butyric acid were detected in the silage with EF9296 strain during the aerobic stability test (Table 3b) in comparison with the control silage.

Table 3a. Bacteria in silage (log cfu/g) inoculated with *Enterococcus faecium* EF9296 strain and in control silage (without inoculant) during the aerobic stability test

Aerobic stability	EF9296 strain	Listeriae	Yeats	Moulds
Day 0–1				
Control silage	ND	6.45 (0.55)	4.19 (0.72)	2.44 (0.19)
EF9296 silage	9.15 (0.15)	4.15 (0.25)*	2.31 (2.31)	3.67 (0.47)
Day 2				
Control silage	ND	4.00 (1.00)	1.77 (0.30)	3.23 (0.23)
EF9296 silage	5.65 (1.65)	1.80 (1.80)	2.75 (1.75)	2.40 (0.80)
Day 5				
Control silage	ND	1.30 (0.30)	3.70 (0.40)	3.15 (0.15)
EF9296 silage	4.73 (0.73)	1.47 (0.00)	5.57 (0.73)	0.00 (0.00)
Day 7				
Control silage	ND	1.73 (0.04)	4.24 (0.07)	4.14 (0.03)
EF9296 silage	4.16 (0.16)	1.38 (0.08)	6.07 (0.23)	2.30 (1.30)

ND = not determined

Table 3b. Chemical analyses of silage (g/kg) inoculated with *Enterococcus faecium* EF9296 strain and of control silage (without inoculant) during the aerobic stability test

Aerobic stability	pH	Lactic acid	Acetic acid	Butyric acid
Day 0–1				
Control silage	5.26 (0.07)	7.03 (1.19)	4.30 (1.19)	1.34 (0.12)
EF9296 silage	4.28 (0.06)**	21.23 (0.65)**	3.57 (0.64)	0.85 (0.12)
Day 2				
Control silage	4.95 (0.23)	21.62 (0.26)	3.11 (0.91)	0.24 (0.24)
EF9296 silage	4.32 (0.00)	24.09 (2.73)	5.03 (0.09)	0.24 (0.24
Day 5				
Control silage	4.95 (0.23)	14.97 (3.77)	4.12 (0.09)	0.97 (0.00)
EF9296 silage	4.36 (0.00)	22.14 (0.52)	3.65 (0.00)	0.00 (0.00)
Day 7				
Control silage	5.57 (0.96)	13.28 (6.51)	3.39 (1.74)	0.73 (0.24)
EF9296 silage	4.29 (0.12)	19.80 (1.83)	3.20 (0.09)	0.36 (0.12)

**$P < 0.01$ significant differences from the control; the value of SD (standard deviation) is in brackets

DISCUSSION

The success of an inoculant as a silage additive depends on many factors, such as the type and properties of plants to be ensiled, climatic conditions, epiphytic microflora, ensiling technique, and the properties of the inoculant (Henderson and McDonald, 1984). The addition of LAB inoculants during ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of LA, lower pH values at earlier stages of ensiling, and improved forage preservation (Filya et al., 2000). However, in some experiments, the addition of LAB inoculants impaired the aerobic stability of silages (Weinberg et al., 1993). *E. faecium* EF9296 sufficiently colonized the silage during ensiling. The counts of EF9296 as an inoculant in silage are comparable with those reported e.g. by Merry et al. (1995) or Ranjit and Kung (2000). Moreover, in the silage inoculated with EF9296 strain a significant increase in the counts of total enterococci was noted after 7 days of ensiling; Merry et al. (1995) found 10^8 cfu/g of enterococci after 7 days of ensiling and at day 14 the enterococci reached 10^9 cfu/g in silage inoculated with LAB. Various LAB can be found on plant surfaces, which indicates that plants are a natural habitat for certain species. The role of LAB on plants is not known because LAB are found in higher numbers on damaged plant parts than in complete plants (McDonald et al., 1991). Pitt and Leibensperger (1987) summarized the literature concerning the numbers of epiphytic LAB found on forage crops and found the mean value of 3.7 log cfu/g of herbage although in our study LAB were present in substantial numbers in the silages. The counts of *E. coli* and enterobacteriae were reduced in the silage inoculated with *E. faecium* EF9296 strain but they were not eliminated; that means the bacteriostatic effect of EF9296 strain against those bacteria was probably produced. Brudzinski and Harrison (1998) reported that *E. coli* cells might develop acid resistance following the induction of an acid tolerance response and, consequently, they would be able to survive at pH as low as 3.4. Contrary to our results, Weinberg et al. (2004) reported that after 6 days of ensiling, the *E. coli* population reached the counts 8×10^3 cfu/g and after one month the number of *E. coli* decreased below the detectable level in maize silage. Under our conditions, a decrease in the counts of staphylococci was observed in the inoculated silage during the whole ensiling period; it is in agreement with the results reported by Faid et al. (1994). There, the microbial profile of fish waste silage showed the elimination of staphylococci after 10 days of ensiling (less than 1 cfu/g silage). Owens and Mendoza (1985) reported that *Clostridium* and *Staphylococcus* were sensitive to low pH.

In agreement with the studies reported by McAllister et al. (1995) and Kung and Ranjit (2001), the application of *E. faecium* EF9296 accelerated

the post-ensiling decline in pH, increased the LA concentration and reduced the concentrations of AA and BA. As soon as a low pH is achieved after ensiling, the aerobic microorganisms and plant enzymes are inhibited more rapidly, which results in reduced proteolysis (Zahiroddini et al., 2004). After 14 days of ensiling a significant decline of pH was detected in the inoculated silage and the effect of *E. faecium* EF9296 on the value of pH was prolonged until the end of experiment (including the aerobic stability test). The inoculation of silage with homofermentative or heterofermentative LAB revealed the lower value of pH (between 3 and 4) compared to the results of our study (Ranjit and Kung, 2000; Winters et al., 2000; Aksu et al., 2004). For example, in the silage inoculated with a mixture of LAB consisting of *Pediococcus*, *Lactobacillus* and *Enterococcus* spp., the pH value 3.76 was measured after 112 days of ensiling (Zahiroddini et al., 2004). However, this lower terminal pH achieved in inoculated silage was related with lower numbers of LAB compared to the control. It could be stated on the basis of our results that a significant increase in LA concentration was noted due to the EF9296 inoculant. This effect was observed not only during ensiling but also during the aerobic stability test. This finding is in agreement with those reported by Chen et al. (1994) or Aksu et al. (2004). Moreover, when *Lactobacillus plantarum* was used for silage inoculation, a higher increase in the concentration of LA was found (Winters et al., 2000) compared to the results reported by us. On the other hand, the inoculation of silage with EF9296 caused a decline in the concentrations of AA and BA in silage. However, AA acts as the growth inhibitor of spoilage organisms by decreasing the maximum growth rate and therefore AA increases the aerobic stability exponentially (Holzer et al., 2003). Filya (2003) found that a small amount of volatile fatty acids such as acetic, propionic and butyric inhibits the aerobic yeast and mould growth in silages.

A part of organic matter gets lost in the initial phase of ensiling owing to the respiration of plants and during silage fermentation and storage by the activity of microorganisms to carbon dioxide and other fermentation products (McDonald et al., 1991). Dry matter losses from fermentation for an optimal lactic acid fermentation are relatively low and should range between 2% and 6% of dry matter (Holzer et al., 2003). In our experiment, a significant increase in DM content in the inoculated silage was detected during the whole ensiling period ($P < 0.001$). Similarly, Henderson (1993) and Weinberg and Muck (1996) reported that the treatment of silages with LAB inoculants could improve DM recovery by 1–3%. Furthermore, in barley silage treated with a mixture of bacterial inoculants (*Pediococcus*, *Lactobacillus* and *Enterococcus* spp.) and enzymes the improvement in DM recovery was found as well (Zahiroddini et al., 2004). Moreover, Khuntia and Chaudhary (2002) reported that dietary addition of a mixed culture of LAB increased DM intake of silage, live weight gain and DM digestibility in calves.

When the silo is opened for feeding, the silage is exposed to aerobic conditions, which leads to its deterioration (Weinberg and Muck, 1996). Indicators for these spoilage processes are increasing temperature and pH, dry matter losses, surface mould growth and feed refusal by the animal (Holzer at al., 2003). Aerobic deterioration of silages is caused by rapid increases in yeasts and mould flora that oxidize lactic acid and volatile acids and result in increased temperatures and pH (Moon, 1983). The inoculation of silage with EF9296 strain led to a reduction of yeasts during the ensiling period, while during the aerobic stability test a decrease in moulds was determined. Most yeasts can grow within the pH range 3–8 and some strains are able to withstand acidities of pH 2 or below (McDonald et al., 1991). In agreement with our results, when the silage was exposed to air, the yeast counts of control and silage inoculated with *L. plantarum*, *E. faecium* and *Pediococcus acidilactici* increased dramatically (Meeske et al., 1999). In addition, the same authors reported that no mould was detected in the inoculated silage after day 5 of ensiling when the pH was 4.4. When exposed to air, high mould counts (10^6 cfu/g of silage) were observed in the control silage while no mould was detected in the inoculated silage (Meeske et al., 1999). McAllister et al. (1998) reported that after 15 days of aerobic exposure, no moulds were detected in the lucerne silage inoculated with *L. plantarum* and *E. faecium*. Besides yeasts and moulds, the other contaminants associated with aerobic spoilage activity in silage are *Bacillus* spp. and *Listeria* spp. (McDonald et al., 1991). The use of silage for animal feeding was sometimes associated with pathological problems, including listeriosis (Wiedeman et al., 1996). Because *Listeria* can grow at low temperatures (Tienungoon et al., 2000), hay silage stored in large plastic bags was frequently contaminated (Wilkinson, 1999). Concerning the counts of

Bacillus and *Listeria* colonies in our experiment, a decrease in bacilli during the ensiling period and a decrease in *Listeria* at the end of ensiling and after 2 days of aerobic exposure were observed. In addition, an inhibitory effect against *Listeria innocua* LMG13568 due to the EF9296 strain in rumen fluid was reported by Marciňáková et al. (2004). It confirmed the antilisterial effect of enterococci and/or their bacteriocins. Moreover, the other bacteriocins produced by *E. faecium* e.g. enterocin A produced by *E. faecium* EK13 strain Mareková et al. (2003) or enterocin M produced by *E. faecium* AL41 reduced *Listeria innocua* cells (Lauková and Mareková, 2002; Lauková et al., 2003).

The EF9296 strain was stable in the silage ecosystem and a reduction in spoilage microorganisms was found especially at days 7 and 14 of ensiling. Moreover, the counts of *Listeria* and moulds were reduced during the aerobic stability test. On the contrary, no influence on yeasts during aerobic exposure was observed. It might be explained by the lack of AA in inoculated silage; that means its amount is not sufficient to prevent the yeast growth. Furthermore, the EF9296 strain influenced pH in silage and increased the amounts of lactic acid. It can be concluded that the silage inoculant *E. faecium* EF9296 enhanced the fermentation and retention of nutrients (by increasing DM content) during ensiling as well as during the aerobic stability test of silage. That means this probiotic and bacteriocinogenic strain, originating from silage, is a promising additive for further improvement of silage quality and/or for its protection against the spoilage microflora.

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