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Effects of Biological and Chemical Additives on Fermentation Progress in Maize Silage

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ABSTRACT

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The objective of this study was to evaluate the effects of bacterial and chemical additives on the number of lactic acid bacteria (LAB) and on fermentation indicators in whole maize silage at 1, 3, 5, 10, and 90 days of fermentation. Maize forage was harvested at approximately 34% dry matter (DM) and treated with (1) no additive (control; C); (2) bacterial inoculant (2 g/t of forage; B) containing the homofermentative LAB *Lactobacillus plantarum, Lactobacillus paracasei*, and *Pediococcus pentosaceus* (1.5×10^{11} cfu/g of inoculant); and (3) chemical additive (4 l/t of forage; CH) containing formic acid, propionic acid, ammonium formate, and benzoic acid. Both treatments decreased pH of silage at day 1 of ensiling ($P < 0.05$), and the lowest value of 4.34 was observed in the CH-treated silage. All silages were well fermented and had pH < 4.0 by day 10 of fermentation. The concentration of lactic acid and the lactic acid : acetic acid ratio increased over time in all treatment groups, and the highest values were 87.5 and 3.62 g/kg of DM, respectively, observed for group B at day 90 ($P < 0.05$). The concentrations of water-soluble carbohydrates were higher ($P < 0.05$) for CH compared to C and B at days 3, 5, 10, and 90 of fermentation. The CH silage had fewer LAB ($P < 0.05$) than did either C or B silages regardless of the days of fermentation. Both additives used in the present study improved fermentation dynamics of the whole crop maize silage.

Keywords: forage conservation; lactic acid bacteria; formic acid; fermentation dynamics

Maize silage is an important source of forage for ruminants in the Czech Republic. It is a highly digestible and palatable feed source valued for its nutritional composition. A well-fermented silage is readily consumed by animals and may improve their health and production characteristics (Varadyova et al. 2010). Ensiling is a method of long-term preservation and storage of fresh plant material under anaerobic and acidic conditions. The primary acid responsible for decreasing the pH of silage is lactic acid, which is produced by lactic acid bacteria (LAB) from water soluble carbohydrates (WSC). LAB occur in varying quantities throughout the natural environment. Although it is well recognized that epiphytic LAB play an important role in silage fermentation, the number of epiphytic LAB in the standing crop is limited and variable (Muck 1990; Lin et al. 1992). In view of the facts that the epiphytic microflora of fodder crops varies greatly and that LAB numbers are usually relatively low, it is very important to know their composition and structure because such knowledge enables successful application of microbial preservative additives (Cai et al. 1998). Their absolute and relative numbers might be important in predicting fermentation adequacy and in deciding whether or not to apply a silage bacterial inoculant (Lin et al. 1992).

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Various types of microbial additives can be used to improve silage fermentation (Reich and Kung 2010). Most commercially available inoculants contain homofermentative LAB, which are used with the objective of stimulating the rate and extent of fermentation so that either the concentration or the proportion of lactic acid in the total fermentation acids in the silage is as high as possible (Jalc et al. 2009; Wilkinson and Davies 2013).

Chemical additives are added to ensiled forages to prevent or reduce the growth of such undesirable microorganisms as yeast or moulds, which are responsible for aerobic deterioration in silages. Inorganic acids, such as formic or sulfuric acids, have been used to improve silage preservation by direct acidification, whereas organic acids, such as propionic, benzoic, and sorbic acids, have been used to increase silage aerobic stability (Kleinschmit et al. 2005; Queiroz et al. 2013). Chemical-based additives are useful for improving fermentation during unfavourable climatic conditions. They can be used when the dry matter (DM) content of ensiled matter is low (e.g. often during rainy weather), for high-protein fodder plants, or for silage with very high DM content (Huhtanen et al. 2013). Because of its high antimycotic activity, propionic acid usually constitutes the greatest percentage of those active ingredients used in commercial products today (Kung et al. 1998; Mills and Kung 2002). However, the nature and intensity of the effect of these additives may differ across plant species and with advancing stage of maturity (McEniry et al. 2014).

Only a few studies have simultaneously compared chemical and bacterial additives used to improve silage preservation (Queiroz et al. 2013). In addition, changes in fermentation characteristics after storage periods of different lengths may bring novel insights into the understanding of fermentation dynamics. During the several first days of fermentation the rate of acidification is important not only with regard to inhibiting undesirable aerobic enterobacteria, yeasts, and some lower fungi, but also due to the fact that it helps increase the production of lactic acid and, thereby, to reduce the degradation of crude protein to ammonia (Dolezal and Zeman 2005). The objective of this study, therefore, was to evaluate the effects of bacterial and chemical additives on the number of LAB and on fermentation indicators in whole maize silage at 1, 3, 5, 10, and 90 days of fermentation.

MATERIAL AND METHODS

Maize (Ronaldinio hybrid; FAO 240/250) was harvested at whole-plant DM content of approximately 33.6% and chopped using a conventional forage chopper to average length of ca. 12 mm for ensiling in a conventional silo. Approximately 60 kg of forage was randomly collected, it was thoroughly mixed, and pre-ensiling samples were taken for analyses. Three piles, each containing approximately 20 kg of forage, were prepared and treated without any additive (C), with a commercial biological inoculant (B), or with a chemical additive (CH). The bacterial inoculant (supplied by Bioferm CZ, Czech Republic), added at 2 g/t of forage, contained the homofermentative LAB *Lactobacillus plantarum, Lactobacillus paracasei,* and *Pediococcus pentosaceus* at total concentration 1.5×10^{11} cfu/g of inoculant. The inoculant (0.04 g) was diluted in 80 ml of distilled water and applied by spraying onto the 20 kg forage during mixing. The chemical additive (Kemira Chemical Oy, Finland) containing formic acid (42.5%), propionic acid (10.0%), ammonium formate (30.3%), and benzoic acid (2.2%) was applied at the rate of 80 ml per 20 kg of forage (4 l/t). The equivalent amount of water was applied to the untreated control forage. Chopped forage samples (700 g; $n = 25$ from each treatment) were packed into polyethylene bags (300 × 400 mm) (Krejčí Packservis Ltd., Czech Republic), vacuum sealed using a VacSy® system (Zepter International Ltd., Czech Republic), and stored in a tempered dark room at +20°C. Silages ($n = 5$ from each treatment) were analyzed for fermentation quality after 1, 3, 5, 10, and 90 days of preservation. The quantity of forage loss on a DM basis was measured after 90 days of preservation.

Chemical analyses of fresh forage and silages were performed in duplicate. The DM content in fresh forage and silage was determined by oven-drying at 105°C for 24 h. For the analysis of chemical composition of plants and silages, samples were oven-dried for 48 h at 50°C and then ground to pass through a 1 mm sieve. Ash was measured after 6 h at 550°C and fat was determined after a 2-hour extraction with petroleum-ether using a Soxtec 1043 extraction unit (FOSS Tecator AB, Sweden). Nitrogen in forage was determined according to the Kjeldahl method (Kjeltec 2400 Analyser, FOSS Tecator AB), and crude protein (CP) was calculated as N × 6.25. A Fibertec™

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2010 (FOSS Tecator AB) was used to analyze fibre content according to AOAC (2005).

Fresh plant and silage pH were determined from aqueous extract (mixture of 100 g of material with distilled water up to volume of 1000 ml) using an InoLab pH 730 pH meter (WTW, Germany). Lactic acid, acetic acid, and butyric acid were analyzed according to Kvasnicka (2000) on an Ionosep 2003 analyser (RECMAN - laboratory equipment, Czech Republic). Titratable acidity (TA) of aqueous extract was detected by alkalimetric titration to pH 8.5 with 0.1 M potassium hydroxide in the presence of formaldehyde. WSC content was determined according to EEC method (EEC 1971), and ammonia N (NH3-N) was analyzed using a Libra S 22 spectrophotometer (Biochrom Ltd., UK) using Nessler's reagent (AOAC 2005).

Forage and silage extracts were prepared by adding 10 g of each sample into 90 ml of 0.5% bacteriological peptone water (Oxoid, UK) and homogenized for 2 min. Then 10-fold dilution series were made by transferring 1 ml aliquots from each separate preceding dilution into 9 ml of 0.5% bacteriological peptone water (Oxoid) to make a corresponding succeeding dilution. The dilutions (0.5 ml of each dilution) were introduced using a sterile pipette to Petri dishes onto Rogosa agar medium (Oxoid CM627). Anaerobic conditions were ensured by pouring over these another layer of the Rogosa agar medium. Petri dishes were incubated at 37°C (INCUCELL-V 111; BMT a.s., Czech Republic) for 72 h to enumerate LAB in fresh maize before ensiling and in silages at 1, 3, 5, 10, and 90 days of the fermentative process.

Statistical analyses were performed using the GLM Procedure of SAS software (Statistical Analysis System, Version 9.1, 2006). The model used for DM loss involved the fixed effect of the additive treatment whereas the model used for the remaining characteristics involved the fixed effects of the additive treatment, days of fermentation, and the interaction of additive treatment × days of fermentation. The slice option was used to test the effect of additive treatment within each day of fermentation and to test the effect of day of fermentation within each additive treatment. When $P < 0.05$, the differences between means were considered significant and were evaluated by Tukey's test. Pearson's correlation coefficient was calculated to evaluate the relatedness of titratable acidity and the sum of lactic and acetic acids concentrations using the CORR procedure of SAS. The data in Table 2 are presented as Least Squares Means (LSM) and standard errors of the mean (SEM; $n = 5$).

RESULTS AND DISCUSSION

The DM content, chemical composition, pH, and number of LAB in the fresh maize forage before ensiling are given in Table 1. The DM content and values of other nutritive constituents were within ranges reported previously for whole maize, whereas the epiphytic LAB number was lower (Reich and Kung 2010; Contreras-Govea et al. 2013; Queiroz et al. 2013). The number of epiphytic LAB on fresh plants is highly variable, ranging from less than 10 to 10^4 cfu/g, and it depends on crop species, climatic conditions, stage of maturity, and the chopping process (Lin et al. 1992).

The fermentative characteristics of maize silage after 1, 3, 5, 10, and 90 days of fermentation are presented in Table 2. All maize silages were well preserved as indicated by the low pH and by the fact that no butyric acid was detected in either control or treated silages. According to Weissbach (1996), the pH values required for the stability of silage at 150, 250, 350, and 450 g DM/kg are 4.10, 4.35, 4.60, and 4.85, respectively. Furthermore, the growth of most acid-tolerant clostridia will be inhibited by a pH just below 5.0 (Jonsson 1991). The pH values observed in the present study were well within this range.

Interactions between treatment and day of fermentation were detected for all the observed characteristics except for DM, thereby indicating that

Table 1. Dry matter content, chemical composition, pH, and the number of lactic acid bacteria (LAB) (± standard deviation) in maize before ensiling

Dry matter (g/kg of fresh matter)	336.3 ± 3.5
Crude protein (g/kg of DM)	78.8 ± 2.9
Crude fibre (g/kg of DM)	191.1 ± 3.8
Ash (g/kg of DM)	46.2 ± 7.1
Fat (g/kg of DM)	39.5 ± 2.1
WSC (g/kg of DM)	125.3 ± 5.7
pH	5.8 ± 0.06
LAB number (log cfu/g of fresh matter)	2.3 ± 0.04

DM = dry matter, WSC = water-soluble carbohydrate

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changes over time depended on the additive used. Neither additive treatment nor day of fermentation affected the DM content ($P > 0.05$). Similarly, no significant differences between treatments in DM loss were observed after 90 days of fermentation (4.0, 1.8, and 3.3 for C, B, and CH, respectively). Significant differences in pH values between treatments were only found at day 1, whereas these were similar at any other time of fermentation. At day 1, the pH was the highest for C, followed by the B treatment, and the lowest pH was detected for CH. This confirmed that added acids are more effective than natural fermentation, because acidification occurs almost immediately after adding the additive (Charmley 2001). The pH gradually decreased from day 1 to day 10 and then remained constant. Significant pH reductions ($P < 0.05$) were particularly observed between days 1 and 3 and between days 3 and 10 for all treatments. The pH values at day 1 correspond with the different

Table 2. Characteristics of maize silage at 1, 3, 5, 10, and 90 days of fermentation

	Treatment	Day of ensiling					Significance			
		1	3	5	10	90	SEM	T	D	T × D
Dry matter (g/kg)	C	339.0	334.2	333.5	333.7	330.3	5.17	ns	ns	ns
	B	342.0	337.4	335.7	354.3	337.6				
	CH	343.3	343.7	341.4	335.6	329.5				
pH	C	4.97^{aX}	4.35^{b}	4.06^{c}	3.91^{c}	3.93^{c}	0.05	***	***	***
	B	4.68^{aY}	4.17^{b}	4.01^{bd}	3.78^{cd}	3.83^{cd}				
	CH	4.34^{aZ}	4.20^{b}	4.10^{b}	3.84^{c}	3.84^{c}				
Lactic acid (g/kg DM)	C	12.8^{aX}	18.9^{a}	35.4^{bX}	47.6^{c}	57.6^{dX}	1.49	***	***	***
	B	17.4^{aXY}	20.0^{a}	39.4^{bXY}	45.4^{c}	87.5^{dY}				
	CH	20.3^{aY}	21.9^{a}	44.0^{bY}	49.0^{b}	59.4^{cX}				
Acetic acid (g/kg DM)	C	14.7^{aX}	18.5^{bX}	21.9^{c}	24.2^{c}	24.2^{cX}	0.72	***	***	**
	B	18.5^{aY}	22.2^{bY}	22.5^{b}	22.9^{b}	24.8^{bXY}				
	CH	21.4^{aY}	23.0^{aY}	23.7^{ab}	25.6^{bc}	29.0^{cY}				
LA/AA	C	0.88^{a}	1.03^{a}	1.62^{b}	1.97^{c}	2.32^{dX}	0.05	***	***	***
	B	0.95^{a}	0.90^{a}	1.75^{b}	1.98^{b}	3.62^{cY}				
	CH	0.95^{a}	0.96^{a}	1.85^{b}	1.92^{b}	2.08^{bX}				
TA (mg KOH/100 g silage)	C	375.4^{aX}	637.4^{b}	700.9^{b}	982.9^{cX}	1575.2^{dX}	31.4	***	***	***
	B	482.5^{aXY}	710.7^{b}	787.5^{b}	854.7^{bX}	1610.3^{cX}				
	CH	571.9^{aY}	652.2^{a}	655.5^{a}	1157.4^{bY}	1802.8^{cY}				
NH3-N (mg N/100 g silage)	C	11.9	11.8	10.7	12.3^{X}	10.9	0.35	ns	ns	***
	B	11.0	11.7	10.6	11.2^{XY}	12.3				
	CH	12.3^{a}	11.4^{ab}	11.6^{ab}	10.5^{bY}	11.6^{ab}				
WSC (g/kg DM)	C	74.1^{a}	45.2^{bX}	30.4^{cX}	22.2^{cX}	7.7^{cX}	2.49	***	***	***
	B	71.2^{a}	51.3^{bX}	14.9^{cY}	15.6^{cX}	9.1^{dX}				
	CH	79.3^{a}	76.1^{aY}	86.9^{aZ}	85.6^{aY}	49.5^{bY}				
LAB number (log cfu/g silage)	C	6.58^{aX}	8.15^{bX}	8.58^{bX}	8.36^{bX}	7.38^{cX}	0.13	***	***	***
	B	6.85^{aX}	8.53^{bX}	8.77^{bX}	8.49^{bX}	7.42^{cX}				
	CH	2.95^{aY}	4.46^{bY}	5.51^{cY}	7.17^{dY}	6.01^{cY}				

C = control, B = bacterial inoculant, CH = chemical additive, SEM = standard error of the mean, DM = dry matter, WSC = water-soluble carbohydrate, LA = lactic acid, AA = acetic acid, LAB = lactic acid bacteria, TA = titratable acidity, T = significance of treatment, D = day of fermentation, T × D = interaction between T and D, ns = not significant

$^{a–d}$values within a row with different superscripts differ at $P < 0.05$

$^{X–Z}$values within a column with different superscripts differ at $P < 0.05$

$^{**}P < 0.01$, $^{***}P < 0.001$

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concentrations of lactic and acetic acids in the respective treatment groups. The pH drop in this study was less rapid than that observed by Meeske et al. (2002), who found pH values to be less than 4 in both control and inoculated silages already after 2 days of fermentation. Similarly, Queiroz et al. (2013) found that all maize silages treated with various chemical and bacterial additives had pH values at day 3 of fermentation ranging from 3.76 to 3.92. Whereas homofermentative LAB grow optimally at 30–35°C (Kung Jr. 2009), in the present study the silages were stored at the ambient temperature of 20°C. That could explain the less rapid growth of LAB and slightly reduced production of lactic acid at early stages of fermentation.

The concentrations of lactic acid gradually increased over time, reaching their highest values at day 90 in all treatment groups. The most rapid elevation was detected in days 3–5, during which the levels of lactic acid almost doubled. At days 1 and 5, the concentrations of lactic acid from highest to lowest were for CH, B, and C. At day 90, the concentration was the highest for B. Similarly to those of lactic acid, albeit to a lesser extent, the concentrations of acetic acid increased over time irrespective of the treatments. Both additive-treated silages showed higher levels of acetic acid compared to the control at days 1 and 3. At day 90, however, the acetic acid concentrations from highest to lowest were for CH, B, and C.

Similar to our study, acetic acid rather than lactic acid was increased in maize silage by bacterial inoculants, indicating that the homofermentative bacteria in these inoculants did not dominate the epiphytic heterofermentative LAB population during the early stages of fermentation (Queiroz et al. 2013). The authors suggested that residual oxygen could impair the growth rate of some homofermentative bacteria, such as *Lactobacillus plantarum*, at the initial stage of silage fermentation. As indicated by the concentration of lactic acid and the lactic acid : acetic acid ratio in the present study, the improved homolactic fermentation due to bacterial inoculant was not evidenced until day 90 of fermentation. Similarly, Contreras-Govea et al. (2013) found that inoculant containing *Lactobacillus plantarum* strain was successful in increasing the concentration of lactic acid in maize silage during 60 days of fermentation.

The chemical additive used in the present experiment did not affect the concentration of lactic acid but did increase the concentration of acetic acid compared to the untreated silage at day 90 of fermentation. It is suggested that the addition of organic acids and ammonium formate created conditions for subsequent growth of heterofermentative LAB in the later stage of silage fermentation and may have contributed to higher concentrations of acetic acid in chemical additive-treated silages. Similar results were observed for formic acid-treated maize silage at day 60 of fermentation (Baytok et al. 2005). Also, the concentrations of lactic and acetic acids were similar among control and chemical additive-treated high-moisture maize silages at 21 and 90 days of fermentation (Da Silva et al. 2015). In contrast, the addition of formic acid into silage decreased the concentration of acetic acid in grass silage (Kennedy 1990). As reviewed by Huhtanen et al. (2013), the inconsistent results obtained in the fermentation quality of formic acid-treated silages can be explained by the variation of crop characteristics, application rates, and evenness of additive application.

The lactic acid : acetic acid ratio is a good efficiency indicator for silage fermentation (Jalc et al. 2009). This ratio ideally should not be less than 3 : 1, and the higher it is the better (Kung and Shaver 2001). In the present study, this ratio increased over time in all treatments and the highest value of 3.62 ($P < 0.05$) was observed for the bacterial inoculant-treated silage at day 90 of fermentation. This indicates that the bacterial strains contained in the additive used made the fermentation more homofermentative.

Titratable acidity was defined in this study as the amount of base (0.1M KOH) necessary to titrate the pH of a silage sample to 8.5, and this increased over time irrespective of treatments. At days 10 and 90, TA was higher in CH than in the other groups ($P < 0.05$). Similar TA values for maize silage ranging from 1010 to 1050 mg NaOH/100 g silage after 6–8 weeks of fermentation were reported by Steidlova and Kalac (2002). As reviewed by Trulea et al. (2013), a high TA value indicates more extensive fermentation, more acid production, and more stable silage for storage and during feed-out. TA is closely correlated with total acid levels in maize silage (Ward 2000), and that was the case also for our results ($r = 0.87$).

The effects of treatment and day of fermentation were not significant for the concentration of NH3-N in this study. However, an interaction

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between these two effects was detected due to a low NH3-N value observed in the CH-treated silage after 10 days of fermentation. The reason for this remains unclear. Our results suggest that natural proteolytic processes were not affected by the additives used, and that is in contrast with the findings of Queiroz et al. (2013), who reported reduced concentrations of NH3-N in maize silages treated by various bacterial and chemical additives. Low NH3-N contents in silage indicate inhibition of proteolysis during fermentation and consequently the improved efficiency of rumen microbial N synthesis (Nsereko and Rooke 1999).

Water soluble carbohydrates are regarded as essential substrates for the growth of LAB during proper fermentation, and low levels may restrict that growth (Nkosi et al. 2011). The WSC concentrations were rapidly reduced in C and B silages during the first 10 days of fermentation ($P < 0.05$), whereas these remained unchanged for CH. In agreement with Meeske et al. (2002), bacterial additive had no effect on WSC during fermentation, indicating that WSC were utilized at the same rate in untreated and inoculant-treated maize silages. The WSC concentrations were higher ($P < 0.05$) in CH compared to C and B at days 3, 5, 10, and 90 of fermentation. As with our study, treating maize silages with the chemical-based additives has been shown to increase residual WSC concentrations, thus suggesting partial inhibition of fermentation (Kleinschmitt et al. 2005; Da Silva et al. 2015). The WSC which remain in silages that have undergone restricted fermentations constitute a potential source of readily available substrate for the growth of aerobic microflora when the silages are exposed to air during the feed-out period (Wilkinson and Davies 2013).

The numbers of LAB rapidly increased in C and B silages during the first 3 days of fermentation ($P < 0.05$), then remained unchanged until day 10, and by day 90 they were lower than at day 10 of fermentation ($P < 0.05$). Although the number of LAB in the B silage was numerically higher than that in the C silage, no significant differences were detected. In contrast, LAB numbers grew markedly more slowly in the chemical additive-treated silage. The CH silage also had fewer LAB than did either untreated or inoculated silages independent of the days of fermentation. Da Silva et al. (2015) reported similar results for high-moisture maize silage treated with a chemical additive.

CONCLUSION

Whole-plant maize silage fermentation was evaluated at 1, 3, 5, 10, and 90 days after ensiling for the effects of treatment with bacterial and chemical additives. Changes observed over time in most characteristics depended on the additive used. Both treatments decreased pH of silage at day 1 of fermentation. All silages were well fermented with pH < 4.0 after 10 days of fermentation. Addition of bacterial inoculant increased the concentration of lactic acid and improved the lactic acid : acetic acid ratio at day 90 of fermentation. The chemical additive containing formic acid, propionic acid, ammonium formate, and benzoic acid did not affect the concentration of lactic acid and increased the concentration of acetic acid compared to untreated silage at day 90 of fermentation.

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