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INFLUENCE OF BACTERIAL-ENZYME ADDITIVE ON NUTRITIVE VALUE AND MYCOTOXIN CONTAMINATION OF HORSE BEAN, ALFALFA AND OAT MIXTURE SILAGES

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ABSTRACT

The influence of bacterial-enzyme additive on nutritive value and concentration of mycotoxins in mixture silages of horse bean, alfalfa and oat with high dry matter content was studied. The fresh matter was harvested in flowering stage of horse bean and after considerable wilting it was cut to 20 mm particles and stuffed into silage bags. We ensiled the mixture in control variant without additives and in trial variant with liquid additive with following biological constituents: *Lactobacillus plantarum, Pediococcus acidilacti, Lactococcus lactis lactis* and enzymatic constituents: celullase and hemicelullase at a dose of 2 liters per ton. The samples were examined for the nutritive value and mycotoxins content (deoxynivalenol, T-2 toxin, zearalenone and total aflatoxins, fumonisins, ochratoxins) by direct competitive enzyme-linked immunosorbent assay. Application of bacterial-enzymatic additive positively influenced the nutritive value of mixture silage of horse bean, alfalfa and oat by statistically significant higher content of nitrogen free extract and PDIE. In silage mixtures we did not notice any positive influence of application of lactic acid bacteria and fibrolytic enzymes on the content of crude protein and fibre. Zearalenone was the secondary metabolite of microscopic fungi with the highest concentration, followed by T-2 toxin and deoxynivalenol. This study suggests that application of bacterial-enzyme additive was sufficient to inhibit the production of zearalenone, fumonisins and ochratoxins.

KEY WORDS: mixture silage; biological additive; nutritive value; mycotoxin

INTRODUCTION

Silages from difficult ensiling forages represent considerable source of proteins in ruminants' nutrition (Holúbek et al., 2008; Jančovič and Vozár, 2000). One of possibilities to ensure good fermentation process is the application silage additives (Bíro et al., 2006a). Biological silage additives contain microbial components on lactic acid bacteria base. The main fermentation product of homolactic bacteria is lactic acid and their application lowers pH in silages. The pH of silages decreases faster, the ratio of lactic acid and acetic acid is improving in favour of preserving lactic acid (Bíro et al., 2006b; Filya et al., 2007), proteolysis is inhibited (Fraser et al., 2001), which positively influences nutritive and hygienic quality of silages. Biological silage additives, which include besides homofermentation also heterofermentation lactic acid bacteria, increase aerobic stability of silages by higher concentration of acetic acid (Kung et al., 2003). Some biological additives also contain enzymatic compounds, mostly on fibrolytic enzyme base, which increases the proportion of fermentable carbohydrates in ensiling matter due to partial degradation of fibre (Mikolajczak et al., 1998). Application of biological silage additives has also positive effect on inhibiton of microscopic fungi producing mycotoxins, which cause different pathologico-anatomical, histological and physiological changes (Suchý and Straková, 2006). The mycotoxins of the greatest concern are aflatoxins, which are generally produced by *Aspergillus* molds, deoxynivalenol,

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zearalenone, T-2 toxin, and fumonisins, which are produced by *Fusarium* molds and ochratoxins produced by *Penicillium* molds (Whitlow and Hagler, 2004). The occurrence of mycotoxins and other contaminants is the possible source of low animal performance and health diseases of animals (Lukáč et al., 2007).

The aim of this study was to examine the influence of bacterial-enzyme additive on nutritive value and occurence of mycotoxins in mixture silages of horse bean, alfalfa and oat with high dry matter content.

MATERIAL AND METHODS

In farm experiments we ensiled mixture, which was sowed in two steps. In the first step was sowed common oat (*Avena sativa*) variety Flämingsstern (15 kg.ha^{-1}), along with horse bean (*Faba vulgaris*) variety Inovec (250 kg.ha^{-1}), and in the second step alfalfa (*Medicago sativa*) variety Palava (20 kg.ha^{-1}). In ensiling matter the ratio was: oat 20%, horse bean 70% and alfalfa 10%. The mixture was harvested when bean was forming hulls. The experiment was realized in co-operation with VPP SPU, farm Kolíňany. The mixture of fresh matter with average content of dry matter 156,53 g.kg^{-1} was wilted to dry matter content of 528,43 (before ensiling control variant) and to 638,8 g.kg^{-1} (before ensiling trial variant). The difference in dry matter content was caused by necessary time needed to fill the silage bags. Wilted ensiling matter was cut to 20 mm particles and stuffed by pressing into silage bags with length 60 m, diameter 2.44 m and thickness 0.224 mm. We ensiled two variants: control variant (C) without additives and trial variant (A) with addition of biological additive, which contained lactic acid bacteria: *Lactobacillus plantarum*, *Pedicoccus acidilacti*, *Lactococcus lactis lactis* (the bacteria concentration 2x10^{13} cfu.g^{-1}) and enzymatic component: cellulase and hemicellulase, applied in liquid state at a dose of 2 l per tone (after dissolving 2.5 g of powder in 2 l of water). After 3 months of fermentation we took the average samples of silages for determination of nutrient content (as per Regulation no. 2145/2004-100) and concentration of mycotoxins. Mycotoxin content of the silages was determined by direct competitive enzyme-linked immunosorbent assays (ELISA). The samples of horse bean, alfalfa and oat mixture silages were analyzed for six mycotoxins including total aflatoxins (AFL), total fumonisins (FUM), total ochratoxins (OTA), zearalenone (ZON), deoxynivalenol (DON) and T-2 toxin (T-2). Samples of silages were dried at 50şC (for 20 hours) and grounded to a fine powder. Extraction of samples was carried out in distilled water (DON), in methanol:water (70:30 v/v) for FUM, AFL, ZON and 50:50 (v/v) for T-2 and OTA. The Veratox quantitative test kits (Neogen, USA) were used and the ELISA procedure performed following the manufacturer's recommendations. The basic principle of the test is the antigen-antibody reaction. The wells in the microtitre plates were coated with antibodies to each mycotoxins. By adding standards of each mycotoxins or the sample solutions the antibody binding sites were occupied proportionate to each mycotoxin concentration. Any remaining free binding sites were occupied in the next stage by enzyme labeled toxin (enzyme conjugate). Any unbound enzyme conjugate was then removed in a washing step. Enzyme substrate and chromogen were added to the wells and incubated. Bound enzyme conjugate converted the colourless chromogen into a blue product. The addition of the stop reagent leaded to a colour change from blue to yellow. Absorbance was determined using the microwell strip reader (Neogen, USA) at 650 nm. A calibration curve for the standards for each toxin dilution was plotted using standard concentration against the percentage inhibition of the standards. Through the use of a microwell reader, the tests provided sample results in µg.kg^{-1} for all mycotoxins. Concentrations of mycotoxins were found out in two replications for each sample of HMC silage. The results were statistically processed using one-factorial variance analysis (ANOVA) of SAS. Means were separated using LSD multiple range test.

RESULTS AND DISCUSSION

In mixture silages of horse bean, alfalfa and oat we detected the content of dry matter 501.0 g.kg^{-1} (C) and 623.6 g.kg^{-1} (A) after termination of the fermentation process. In silages with biological additive (A) we detected statistically significant lower content of crude protein, which also confirmed the results of Krzywiecki et al. (2003). Doležal (2002) confirmed positive influence of biological additives on the content of N-compounds in silages from difficult ensiling forages. In mixture silages of variant C we detected fat content of 14.4 g.kg^{-1} of DM, while in variant A fat content was statistically non-significantly lower at 0.5 g.kg^{-1} of DM. Mixture silages were characterized by high content of fibre as a consequence of late harvesting. Values of fibre contents in both variants were nearly identical (361.0 and 361.4 g.kg^{-1} of DM) without statistically significant differences. Petrikovič et al. (2000) determined average content of fibre in silages from horse bean, alfalfa and oats at 352 g.kg^{-1} of DM. In the experiment we did not detect any positive effect of addition of cellulase and hemicellulase on fibre content, which also confirmed the results of Kung et al. (1991). On the other hand, Nedeau et al. (2000) and Rajčáková and Mlynár (2006) determined a decrease of fibre fractions after application of cellulases. In silages with biological additive (A) we determined lower content of ash, which positively affects organic matter content. In variant A silages we detected in comparison with control variant C statistically significant higher content of nitrogen free extract at 20.9 g.kg^{-1} of DM. Kung et al.

Table 1: Nutrient content in mixture silages of horse bean+alfalfa+oat

variant		DM	CP	F	CF	A	NFE	OM
		g.kg^{-1}	g.kg^{-1} of DM					
C	$\bar{x}$	501.0^{a}	116.6^{a}	14.4	361.4	109.5	396.6^{a}	889.1
	s	35.729	2.787	0.643	5.147	2.397	2.702	2.397
A	$\bar{x}$	623.6^{a}	108.6^{a}	13.9	361.0	99.0	417.5^{a}	901.0
	s	7.197	1.153	1.242	6.536	7.601	4.029	7.601

DM-dry matter, P-crude protein, F-fat, CF-crude fibre, A-ash, NFE-nitrogen free extract, OM-organic matter, C-control variant (without additive), A-bacterial-enzyme additive, $\bar{x}$ -average, *s*- standard deviation, values with identical superscripts in columns differ significantly at P<0.05

Table 2: Energy and nitrogen content of mixture silages of horse bean+alfalfa+oat

variant		NEL	NEG	PDIN	PDIE
		MJ.kg^{-1} of DM		g.kg^{-1} of DM	
C	$\bar{x}$	4.89	4.58	70.9^{a}	61.2^{a}
	s	0.015	0.012	0.503	1.686
A	$\bar{x}$	4.97	4.67	66.07^{a}	62.93^{a}
	s	0.053	0.057	0.681	0.902

NEL-net energy of lactation, NEG-net energy of gain, PDIN and PDIE: protein digestible in intestine of ruminants values with identical superscripts in columns differ significantly at P<0.05

(1991) did not detect positive effect of enzymes addition on content of fermentable carbohydrates in alfalfa silages. Mixture silages of horse bean, alfalfa and oat with addition of biological additive were characterized by statistically non-significant higher energetic value: NEL (4.97 MJ.kg^{-1} of DM) and also NEG (4.67 MJ.kg^{-1} of DM). Higher NEL value in variant A represents difference of 0.08 MJ.kg^{-1} of DM in comparison with variant C. Lád et al. (2008) identically detected statistically non-significant higher energetic value in silages with bacterial-enzymatic additive. Petrikovič et al. (2000) determined average energetic value in silages of horse bean, alfalfa and oat: 4.38 MJ NEL and 3.99 MJ NEG in 1kg of DM. Content of digestible protein in intestine of ruminants were determined in silages: A variant accounted for lower value of fraction PDIN, as a consequence of lower content of N-compounds, but higher value of PDIE in comparison with C variant silages. Differences in both fractions were statistically significant.

The analytical results for mycotoxins indicate that samples of mixture silages were contaminated with all the determined mycotoxins. After 3 months of storage the most prevalent mycotoxin was ZON, followed by T-2 toxin and DON. ZON was detected at concentrations of 336.4 (A) and 992.7 µg.kg^{-1} (C). Differences in ZON concentrations were statistically significant (P<0.05). Nedělník and Moravcová (2006) in Czech Republic analyzed the average concentration of ZON in alfalfa silages (577 µg.kg^{-1}) and lower value of 500 µg.kg^{-1} in cereal silage (barley). Zearalenone is the cause of hyperestrogenism, earlier sexual maturity, vaginitis, prolonged oestrus and interfertility (Palti, 1978) and sheeps become sterile (Towers and Sprosen, 1993). Intake of T-2 toxin can significantly retard the folliculus maturation and ovulation and perhaps the subsequent luteinisation also in ruminants (Huszenicza et al., 2000). The lowest content of T-2 toxin was determined in control variant of mixture silages (211.9 µg.kg^{-1}) and the highest T-2 toxin concentration was identified in trial variant of mixture silages treated with bacterial-enzyme additive (213.9 µg.kg^{-1}). T-2 toxin is associated with reduced immunity, blood coagulation problems and heamorrhage (Nedělník and Moravcová, 2006). Deoxynivalenol is one

Table 3: Concentration of mycotoxins in mixture silages of horse bean+alfalfa+oat

variant		ZON	T-2	AFL	FUM	OTA	DON
		µg.kg^{-1}					
C	$\bar{x}$	992.7^{a}	211.9	6.3^{a}	142.8^{a}	7.0^{a}	183.1
	s	9.687	3.026	0.071	1.174	0.057	1.683
A	$\bar{x}$	336.4^{a}	213.9	7.3^{a}	32.1^{a}	2.89^{a}	174.6
	s	1.230	5.713	0.113	0.467	0.028	2.984

ZON-zearalenone, T-2-T-2 toxin, AFL-total aflatoxins, FUM-total fumonisins, OTA-total ochratoxins, DON-deoxynivalenol, values with identical superscripts in columns differ significantly at P<0.05

of the most common mycotoxins found in feeds. DON is associated with nausea, vomiting, diarrhoea, weight loss and food refusal (Rotter et al., 1996). In the present study, the samples of control mixture silages had higher mean level of DON (183.1 mg.kg^{-1}), while the samples of A variant contained the mean level of 174.6 mg.kg^{-1}. Higher DON values were observed by Bíro et al. (2007) in alfalfa silages (200-300 µg.kg^{-1}). Concentrations of T-2 toxin and DON in treated silages were not significantly different in comparison with control. Our results did not confirm previous findings that selected strains of *Lactobacillus* are able to remove T-2 toxin and deoxynivalenol (El-Nezami et al., 2002). The lowest contamination of mixture silage samples was by AFL and OTA mycotoxins. Significantly higher content ($P<0.05$) of AFL was found in silages treated with bacterial-enzyme additive than in untreated silages. Results of Hassan and Lloyd (1995) showed that isolates of *Lactobacillus* had a slight effect on mold growth and aflatoxin production. In acute clinical aflatoxicosis, signs of acute hepatic injury are seen as coagulopathy, increased capillary fragility, haemorrhage, and prolonged clotting times (Pier, 1992). Application of additive positively affected ($P<0.05$) the concentration of OTA. In agreement with our results, Cabo et al. (2002) reported apparent antifungal activity of several lactic acid bacteria against genera *Penicillium*, which is potential producer of ochratoxins. In our study it was detected that the content of OTA in concentrations of 2.89 (A) and 7.0 µg.kg^{-1} (C). Ochratoxin A is nephrotoxin, liver toxin, immune suppressant, potent teratogen and carcinogen (Kuiper-Goodman and Scott, 1989). Fumonisins affect animals in different ways by interfering with sphingolipid metabolism (Merrill et al., 2001). They cause leukoencephalomalacia in horses, pulmonary edema in swine and they have hepatotoxic and carcinogenic effects (Rheeder et al., 1992). Our study confirmed that concentrations of FUM were affected ($P<0.05$) by treatment with a commercial preservative based on a mixture of lactic acid bacteria and enzymes.

CONCLUSION

Application of bacterial-enzymatic additive positively influenced nutritive value of horse bean, alfalfa and oats mixture silages by statistically significant higher contents of nitrogen free extract and PDIE, as well as higher contents of organic matter and energetic value, but without statistically significant differences. In mixture silages we did not detect positive influence of addition of lactic acid bacteria and fibrolytic enzymes on the contents of crude protein and crude fibre. Occurrence of observed mycotoxins was detected in all horse bean, alfalfa and oat mixture silages. The results showed that ZON was the secondary metabolite with the highest concentration, followed by T-2 toxin and DON. These data suggesting the application of bacterial-enzyme additive were not sufficient to inhibit the concentration of T-2 toxin and AFL, whereas significantly reduced the concentration of ZON, FUM, OTA and non-significantly, the content of DON.

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