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ORIGINAL PAPERS

EFFECT OF ADDITIVES ON THE FERMENTATION PROFILE, MICROBIAL COLONIZATION AND OXYGEN STRESS OF ALFALFA SILAGES

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Abstract

Alfalfa is a popular source of proteins in diets for ruminants even though the plant is difficult to ensile. The high content of crude protein, high buffering capacity, low concentration of soluble carbohydrates, an undesirable content of nitrate(V) and the presence of epiphytic lactic acid bacteria justify the use of biological or chemical additives when ensiling this raw material. The study was performed to assess the effectiveness of an inoculant and a combined additive (microbiological preparation and potassium sorbate) for alfalfa ensilaging. Wilted green forage was ensiled in 12 mini-silos and stored for 12 weeks. Three variants of silages were prepared: control with no additive and test variants with inoculant and combined additives. The following were determined in the collected samples: chemical composition (green forage and silages), counts of lactic acid bacteria, aerobic mesophilic bacteria, yeasts and moulds (green forage and silages), the level of toxins – aflatoxins, ochratoxin A, toxins T-2 and HT-2, nivalenol, deoxynivalenol, zearalenone (green forage and silages), the quality of silages (pH value, content of alcohol and volatile fatty acids, aerobic stability). The additives clearly have improved the fermentation profile but have not increased the oxygen stability of silages. The highest efficiency was determined for the combined additive, which significantly decreased the pH value, increased the content of lactic acid and acetic acid, reduced the count of aerobic mesophilic bacteria and slightly decreased the count of moulds in relation to the control silage or silage with the inoculant. The only moulds detected silage with the combined additive belonged to *Penicillium*, but the silage with the inoculant contained *Mucorales* beside *Penicillium*. The effect of the combined additive containing a microbiological preparation and potassium sorbate on ensiling alfalfa was stronger than that produced by the inoculant.

Keywords: aerobic stability, alfalfa, bacteria, moulds, mycotoxins, ensiling additives, yeasts.

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INTRODUCTION

Alfalfa silage is often used as a valuable roughage feed in diets for ruminants. However, good quality alfalfa silage is difficult to make due to the high buffering capacity, an unsuitable content of nitrate(V) and a low concentration of soluble carbohydrates, as well as the presence of epiphytic lactic acid bacteria in the raw material (McEniry et al. 2006, Purwin et al. 2006, Kaiser, Weiss 2007, Juráček et al. 2009, Purwin et al. 2009, 2010, 2015, Zielińska et al. 2015). The poor ensiling quality of harvested material can affect adversely the fermentation process, for example by ab intensive growth of facultative aerobic yeasts and acid-resistant moulds and other unwanted microorganisms, which occur on plants naturally. When found in feeds, such microorganisms, primarily moulds, not only pose a threat to the health and productivity of animals, but are also a potential source of contamination of animal products (mainly milk) with various toxins. In ruminants, there is a high probability of multiple contamination because they are fed with roughage and concentrate. Mycotoxins as secondary metabolites of fungi are produced under stress conditions if the humidity, air and temperature promote their appearance (Purwin et al. 2006, Alonso et al. 2013, Cheli et al. 2013).

In order to reduce the risk of producing poor quality alfalfa silage, it is necessary to use silage additives. The most popular are inoculants containing lactic acid bacteria, which stimulate the fermentation process, inhibit the growth of unwanted microorganisms, primarily yeasts and moulds, and promote the aerobic stability of silages, which may improve the fermentation profile and aerobic stability of the feed (Wilkinson, Davies 2012, Zielińska et al. 2015). The strength of additives depends on the species composition of the microflora (Driehuis et al. 1999, Danner et al. 2003, Nkosi et al. 2011, Alonso et al. 2013, Cheli et al. 2013, Jatkauskas et al. 2013). The latest generation of biological additives comprises inoculants containing several types and strains of bacteria (Nkosi et al. 2011, Wilkinson, Davies 2012, Jatkauskas et al. 2013). The combination of homo- and heterofermentative bacteria may be more advantageous than single strains because of the ability to function in a wider range of pH, positive mutual interactions, higher production of lactic acid as well as acetic acid generation and improved aerobic stability of the silage (Jatkauskas et al. 2013). However, heterofermentation is less efficient in terms of acidification and protection of nutrients in the anaerobic phase. Literature shows that an alternative to heterofermentative bacterial inoculants is a combination of homofermentative bacteria and salts such as sorbic, benzoic, propionic and sulphuric(VI) acids. Bacteria stimulate fermentation, whereas organic acid salts reduce the activity of yeasts and moulds, thus improving the aerobic stability of silage (Polan et al. 1998, Kung et al. 2004, Wilkinson, Davies 2012).

The aim of this study was to compare the efficiency of two additives in alfalfa ensiling: an inoculant and a combined additive, in terms of their influence on the fermen tation profile, aerobic stability and hygienic quality.

MATERIAL AND METHODS

Test material

The second cut of cv. Alba alfalfa (*Medicago sativa* L.) was harvested in the initial stage of blooming. After chopping to an average length of 30 mm, wilted green fodder was ensiled in 12 PVC mini-silos, each of a capacity of 8654 cm^3 (diameter 15 cm, height 49 cm) and equipped with a gas valve. The ensiled biomass was compacted in each mini-silo, with the use of a specially designed pneumatic tool for plant material pressing, to achieve the density of 250 kg dry matter per m^3. The pressing force was adjusted through the stabilization of a pressure supplying injector plunger set with a reducer. The pressing pressure of the injector during the pressing reached 6×10^5 Nm^{-2}.

Three silage variants with 4 replications were prepared: control with no additive (C), with an inoculant (I) and with a combined additive (CA), which consisted of two parts: microbiological and chemical one. The inoculant (I) contained the following bacteria: *Enterococcus faecium CCM 6226, Lactobacillus casei CCM 3775, Lactobacillus plantarum CCM 3769, Pediococcus spp. CCM 3770* at an amount of of 1×10^{10} cfu g^{-1} of the preparation and was used at a dose of 0.01 kg t^{-1} of the ensiled material. The additive was dosed as water solution at a dose of 2 dm^3 t^{-1} of forage (after dissolving 0.01 kg inoculant in 2 dm^3 of distilled water). The microbiological part of the combined additive (CA) contained the following bacteria: *Pediococcus acidilactici* 33-06, NCIMB 30086, *Pediococcus acidilactici* 33-11, NCIMB 30085*, Enterococcus faecium* M74, NCIMB 11181*, Lactobacillus plantarum* L-256, NCIMB 30084 at an amount of 2.0×10^{11} cfu g^{-1}, and the chemical part was composed of potassium sorbate. The microbiological and chemical parts of this additive were added at doses of 0.002 and 0.2 kg t^{-1} of the ensiled forage, respectively. The additive was dosed as water solution at 2 dm^3 t^{-1} of forage (after dissolving 0.002 kg microbiological part and 0.2 kg potassium sorbate in 2 dm^3 distilled water). In order to ensure comparable ensiling conditions, forage without additives was sprayed with the same volume of distilled water. The water and additives were sprayed onto fresh forage from a spray bottle and the material was carefully mixed. The mini-silos were stored for 12 weeks in an air-conditioned room at 20°C.

Sampling and chemical analysis

The samples of green forage and silages were collected for analysis. Green forage underwent determinations of dry matter, crude ash, crude protein, neutral detergent fibre (NDF), acid detergent fibre (ADF), water soluble carbohydrates (WSC), nitrates(V) (AOAC 1990) and buffering capacity (Weissbach 1992). The fermentation coefficient (FC) was calculated (Pahlow, Weissbach 1999).

In silages, the following were determined: pH value (pH-meter N-517), content of dry matter and ammonia-N (AOAC 1990), ethanol and organic acids (lactic, acetic, propionic, butyric, isobutyric, valeric and isovaleric) by HPLC with refractometric detection using a Merck-Hitachi system consisting of a pump L-7100, autosampler L-7200, column oven L-7300, refractometric detector L-7490. Ethanol and acids were separated on a chromatographic column ORH ICSep ICE-801 using the mobile phase 2.5 mM of sulphuric(VI) acid. The dry matter content in silages was corrected (cDM) for volatile compounds (Weissbach, Kuhla 1995).

Aerobic stability test

Aerobic stability of all silages was tested for 7 days in an air-conditioned room at ambient temperature of 20±1°C (Honig 1985). Silage temperatures during the stability test were recorded at 1 hour intervals (Squirrel 2000).

Microbiological and mycotoxicological analysis

Samples for microbiological assays were prepared according to PN-EN ISO 6887-1:2000. Counts of lactic acid bacteria (LAB) were determined on MRS agar (Merck) after anaerobic incubation (20% CO_2) at 30°C for 72 h (PN-ISO 15214: 2002, with own modification). Counts of aerobic mesophilic bacteria (AMB) were checked on an agar medium (5 g of meat peptone, 3 g of meat extract, 15 g agar (Merck), pH 7.2, after 72 h of aerobic incubation at 30°C (PN-R-64791: 1994). Qualitative and quantitative identification of moulds and yeasts followed the norm PN-ISO 7954:1999 with own modifications. Mycological examination of silages was carried out on an YGC agar medium (yeast extract, glucose with 100 ppm chloramphenicol), 5-7 days incubation at 25±1°C. Identification of moulds was performed to genus. The results are expressed as colony forming units (cfu) per gram of a sample.

Trichothecenes and zearalenone

A sample (12.5 g) was homogenized with 50 ml of ACN:H_2O (80:20) for 3 minutes. The filtered extract (4 ml) with 40 µl of ZAN (c = 1000 ng ml^{-1}) solution was applied onto a Bond Elut® Mycotoxin column (Agilent, Santa Clara, CA, USA). Subsequently, the mixture of purified extract (2 ml) with 50 µl of internal standard solution (13 C-DON; c = 2500 ng ml^{-1}) was evaporated to dryness using nitrogen. MeOH:H_2O (1:4) (495 µl) was added to the vial and the sample was vortexed. Trichothecenes and ZEN were determined using HPLC with MS/MS detection. HPLC: Agilent 1200 (Agilent Technologies Inc., Santa Clara, CA, USA), mass spectrometer: 3200 QTRAP (AB Sciex, Foster City, CA, USA), chromatographic column: Gemini C18 (150 × 4.6 mm, 5 µm) (Phenomenex Inc., Torrance, CA, USA), mobile phase: A: H_2O + 5 mM CH_3COONH_4 + 1% CH_3COOH, B: MeOH + 5 mM CH_3COONH_4 + 1% CH_3COOH, flow rate: 0.7 ml min^{-1}, injection volume: 20 µl.

Ochratoxin A

A sample (12.5 g) was homogenized with 50 ml of ACN:H_2O (60:40) for 2 minutes. The extract was centrifuged at 4000 rpm for 20 minutes. The mixture of 5 ml of the supernatant with 55 ml of PBS solution was filtered; 48 ml of the diluted extract was applied onto an OCHRAPREP® column (Rhone Diagnostic Technologies Ltd, Glasgow, UK) at a flow rate of 2-3 ml min^{-1}. The column was washed with 20 ml of H_2O and dried with air. Ochratoxin A was eluted using 1.5 ml of MeOH:CH_3COOH (98:2). The eluate was collected into a sample vial, 1.5 ml of H_2O was passed through the column and the sample was vortexed. Ochratoxin A was determined using HPLC with fluorescence detection (FLD). HPLC: LaChrom ELITE (Merck-Hitachi, Darmstadt, Germany), chromatographic column: LiChrospher 100 RP-18 (250 × 4.0 mm, 5 µm), mobile phase: ACN:2%CH_3COOH (70:30), flow rate: 1.0 ml min^{-1}, injection volume: 50 µl.

Aflatoxins

A sample (25 g) with added NaCl (2.5 g) was homogenized with 50 ml of MeOH:H_2O (80:20) for 1 minute. 10 ml of filtered extract was added to 40 ml of H_2O, shaken and filtered again; 10 ml of diluted extract was applied onto an AflaTest® column (Vicam, Watertown, USA). The column was washed twice with 10 ml of H_2O. Aflatoxins were eluted using 1 ml of MeOH. The eluate was collected into a sample vial, 1 ml of H_2O was added and the sample was vortexed. Aflatoxins were determined using HPLC with FLD preceded by post-column derivatization. HPLC: Merck-Hitachi (Darmstadt, Germany), chromatographic column: LiChrospher 100 RP-18 (250 × 4.0 mm, 5 µm), mobile phase: ACN:MeOH:H_2O (20:20:60) + 119 mg KBr + 100 µl 65% HNO_3, flow rate: 1.0 ml min^{-1}, injection volume: 50 µl.

Statistical analysis

The results were statistically processed with SAS/STAT software (SAS, 2009) using one-way analysis of variance according to the model: $Y_{ij} = \mu + a_i + e_{ij}$, where: Y – value of variable, µ – mean, a_i – effect of the group, e_{ij} – experimental error. Significant differences were verified using the least significant difference test. The compatibility of variable distribution with normal distribution was verified with the Kolmogorov-Smirnov test, while the homogeneity of variance was tested with the Brown-Forsythe procedure.

RESULTS AND DISCUSSION

Wilted alfalfa green forage, up to the dry matter content of 293 g, was low in water soluble carbohydrates and nitrates(V), high in protein and had a high buffer capacity at the fermentation coefficient (FC) below 35 (Table 1).

Table 1
Chemical composition and microbial colonization of alfalfa green forage

Variable	Mean	SD
Dry matter (g kg^{-1} FM)	293.0	5.7
Crude ash (g kg^{-1} DM)	114.3	2.0
Crude protein (g kg^{-1} DM)	157.3	4.2
Crude fat (g kg^{-1} DM)	24.6	0.8
NDF (g kg^{-1} DM)	433.9	7.8
ADF (g kg^{-1} DM)	339.4	7.5
WSC (g kg^{-1} DM)	51.5	3.5
BC (g lactic acid 100 g^{-1} DM)	11.44	0.9
FC	32.89	0.6
NO_3 (g kg^{-1} DM)	2.90	0.2
LAB (log cfu g^{-1} FM)	6.0745	0.1186
AMB (log cfu g^{-1} FM)	6.2404	0.1260
Yeasts (log cfu g^{-1} FM)	5.0835	0.2462
Moulds (log cfu g^{-1} FM)	6.0070	0.1980

SD – standard deviation; DM – dry matter; FM – fresh matter; NDF – neutral detergent fibre; ADF – acid detergent fibre; WSC – water soluble carbohydrate; BC – buffer capacity; FC – fermentation coefficient; NO_3 – nitrates(V); LAB – lactic acid bacteria; AMB – aerobic mesophilic bacteria; cfu − colony forming unit

The parameters experssing the suitability of alfalfa for silage justified the use of ensilaging additives (Pahlow, Weissbach 1999, Thaysen 2004, Kaiser, Weiss 2007).

Beside the chemical composition, another important factor affecting the fermentation process is the count of epiphytic LAB. There should be no less than 5 log cfu of these bacteria per g green forage (Pahlow, Weissbach 1999). The count of epiphytic LAB in the tested raw material slightly exceeded 6 log cfu g^{-1}, and was higher than usually reported in literature (Kung et al. 2004, Kizilsimsek et al. 2007).

The green fodder was also colonized by undesirable microorganisms such as mesophilic anaerobic bacteria, yeasts and moulds (Table 1). The mesophilic aerobic bacteriaL content in green forage was by 4% above the acceptable contamination level of feed materials, which is 6 log cfu g^{-1} (Kukier et al. 2012). Literature data (Pahlow et al. 2003) identify the counts of yeasts present on plants as typically between 3 to 5, and the counts of moulds ranging from 3 to 4 log cfu g^{-1} of green fodder. In our study, the ensiled biomass was colonized by these microorganisms more heavily: by about 1.7 to 70% more of yeasts and from 50 to 100% of moulds. The colonization of plants by unwan-

ted microorganisms depends on several factors: the environmental conditions, cultivation system, agronomic practices, etc. (Cheli et al. 2013).

Determination of fungal counts is a way to assess the hygienic quality of feed. It is recommended that the number of fungi (according to good manufacturing practices) in ensiled raw material of good hygienic quality should not exceed 4 log cfu g^{-1} (Alonso et al. 2013).

With respect to the toxicogenic aspect, the fungi that most frequently occur on plants are moulds of the genera *Fusarium* and *Aspergillus*, excreting deoxynivalenol, nivalenol, fumonisin and aflatoxin (Purwin et al. 2006, Alonso et al. 2013). The ensiled alfalfa examined in our study was not colonized by the above fungi, while almost 91% of the detected fungi represented *Dematiaceae* (Table 2).

Table 2

Mould share (%) and mycotoxin content in alfalfa green forage

Moulds	Green forage
Dematiaceae	90.81
Aureobasidium	0.63
Cladosporium	0.92
Oidium	0.04
Mucor	0.14
Penicillium	0.02
Acremonium	0.01
Epicoccum nigrum (Dematiaceae)	0.01
Not identification	7.42

Mycotoxins: T-2 toxin, HT-2 toxin, nivalenol (NIV), deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA), aflatoxins (AF) were not detected in the tested samples of green forage.
Limit of detection (ppb): T-2 – 0,20; HT-2 – 0,73; NIV – 1,0; DON – 1,0; ZEN – 0,07; OTA – 0,13; AFB1 – 0.05; AFB2 – 0.02; AFG1 – 0.25; AFG2 – 0.08

The corrected dry matter content in the silages with an additive (I, CA) was higher than in the control silage (C) – Table 3. The share of ammonia nitrogen in total nitrogen was the same. The ethanol content did not exceed 4 g kg^{-1} dry matter. The additives clearly enhanced the fermentation profile during alfalfa ensiling. The highest efficiency was found for the combined additive (CA), which significantly decreased the pH value and increased the content of lactic acid and acetic acid in comparison to the other silages. The lowest content of acetic acid and propionic acid was in silage with the inoculant (I). A similar relationship was recorded by Jatkauskas et al. (2013), which tested the ensiling of wilted alfalfa (328 g kg^{-1}) with a *Lactobacillus*

Table 3

Fermentation profile, aerobic stability and microbial colonization of alfalfa silages

Variable	C	I	CA	SEM	*P-value*
cDM (g kg^{-1})	297.3^{A}	300,0^{B}	299.6^{B}	0.43	0.0054
pH	4.27^{C}	4.21^{B}	4.16^{A}	0.01	0.0003
N-NH_3 (g kg^{-1} N total)	94.23	95.57	92.53	1.27	0.6663
Ethanol (g kg^{-1} cDM)	3.24^{A}	3.40^{B}	3.54^{C}	0.04	<0.0001
Lactic acid (g kg^{-1} cDM)	60.89^{A}	70.01^{B}	70.77^{C}	1.35	<0.0001
Acetic acid (g kg^{-1} cDM)	34.98^{B}	28.10^{A}	39.05^{C}	1.36	<0.0001
Propionic acid (g kg^{-1} cDM)	2.41^{C}	2.22^{A}	2.40^{B}	0.03	<0.0001
Aerobic stability (h)	168	168	168	–	–
LAB (log cfu g^{-1} FM)	7.1861	7.2353	7.1547	0.0203	0.2856
AMB (log cfu g^{-1} FM)	6.7550^{B}	6.6882^{B}	5.6289^{A}	0.1663	0.0002
Moulds (log cfu g^{-1} FM)	2.8595	2.9556	2.6824	0.0529	0.0886

cDM – corrected dry matter; N-NH_3 – ammonia-N; SEM – standard error of mean; FM – fresh matter; LAB – lactic acid bacteria; AMB – aerobic mesophilic bacteria; cfu – colony forming units; ^{A,B,C} – means with different superscripts within row differ significantly at $P < 0.001$; Butyric, isobutyric, valeric, and isovaleric acids were not detected in silage samples. Yeasts were not detected in 0.01 g.

buchneri inoculant. However, the lactic acid concentration the cited authors recorded was lower than in our study due to the heterofermentative fermentation pathway of these bacteria. Wilkinson, Davies (2012) report that ensilaging raw material with a low content of water-soluble carbohydrate was not significantly affected by inoculants containing LAB in terms of the amount of final fermentation products or the aerobic stability of silages vs control silage.

The temperature of silage exposed to air for 168 h (Table 3) did not exceed the threshold temperature marking the loss of oxygen stability, i.e. 23°C (Pahlow, Weissbach 1999). Jatkauskas et al. (2013) and Zielińska et al. (2015) demonstrated that microbiological additive and microbiological additive with sodium benzoate increased the stability of grass, maize and alfalfa silages. The worse oxygen resistance could have been caused by a lower concentration of acetic acid (up to 21.5 g) than in our study (average content 34 g kg^{-1} cDM). In a study of Wambacq et al. (2013), it was indicated that a microbiological additive increased the aerobic stability of alfalfa with perennial ryegrass silage from 145 to 360 h, with the acetic acid concentration of 30 g. The published data indicate that the content of undissociated acetic acid of more than 8 g kg^{-1} of fresh matter is necessary for the aerobic stability of silage, because it has an inhibitory effect on the growth of yeasts and moulds (Danner et al. 2003, McEniry et al. 2006, Wilkinson, Davies 2012). The antimicrobial activity of acetic acid is high when the pH values of silages are

low (around pH 4), because its major part is undissociated (Danner et al. 2003, Wilkinson, Davies 2012). In our own silages, the acetic acid content ranged from 8.43 (I) to 11.70 (CA) g kg^{-1} of fresh matter at a pH value from 4.21 to 4.16, respectively. The silage additives did not differentiate counts of LAB. The combined additive (CA) significantly reduced the count of AMB in relation to the control silage (C) and silage with the inoculant (I), about 16%, and slightly decreased the count of moulds to 16.67 and 15.84%, respectively. Yeasts were not detected in the silages. The presence of yeasts and moulds in silages defines the hygienic quality of these feeds (Alonso et al. 2013). Among the tested additives, the combined additive (CA) was the most effective towards moulds, although the differences were not significant. Similar but statistically significant correlations were found by Wambacq et al. (2013) in a trial on ensiling alfalfa with perennial ryegrass. Rossi, Dellaglio (2007) as well as Wambacq et al. (2013) claim that counts of yeasts and moulds in alfalfa silage and silage from alfalfa with perennial ryegrass ranged from 0 to more than 4, and from 2.03 to 3.25 log cfu g^{-1}, respectively. In good quality grass silages, the normative concentration of moulds and yeasts should not exceed 3-4 log cfu g^{-1} of fresh matter (McEniry et al. 2006). Theonly moulds found in the silage with the combined additive (CA) were *Penicillium* moulds. In the silage with the inoculant (I), beside *Penicillium* making up over 83% of the total moulds, there were also *Mucorales* (Table 4). However, *Mucorales* were more numerous that *Penicillium* in the control silage (C), with the share of about 32% in the total number of moulds. In our study, the counts of *Penicillium* and *Mucorales* did not exceed the acceptable level, which is 3.6990 for *Penicillium* and *Aspergillus*, and 3 log cfu g^{-1} of silage for *Mucorales* (Wagner et al. 2007). Bauer, Meyer (2006) showed that *Penicillium* and *Mucoraceae* were also dominant in grass silage, while others (Wilkinson, Davies 2012) report that the count of yeasts exceeding 5 log cfu g^{-1} of fresh matter depresses the aerobic stability of silages. The silage samples were not contaminated with any of the mentioned mycotoxins.

Table 4

Mould share (%) and mycotoxin content in alfalfa silages

Moulds	C	I	CA
Mucorales	67.50	16.50	nd
Penicillium	32.50	83.50	100.00

nd – not detected in 0.01 g;
Mycotoxins T-2, HT-2, NIV, DON, ZEN, OTA were not detected in the tested samples of silages. Limit of detection – see Table 2.

Silages exposed to air did not contain yeasts (Table 5). The additives significantly reduced the growth of moulds in silages during the aerobic stability test compared to the control sample (C). The reduction was 23% due to the microbial (I) additives and 35% caused by the combined additive (CA). The

Table 5

Colonization yeasts and moulds of alfalfa silages after aerobic exposition (log cfu g^{-1} FM)

Microorganisms	C	I	CA	SEM	*P-value*
Yeasts	nd	nd	nd	–	–
Moulds (100% *Penicillium*)	4.1420^{C}	3.1921^{B}	2.6981^{A}	0.3210	<0,0001

FM – fresh matter; nd – not detected in 0,01 g; SEM – standard error of mean;
^{A,B,C} – means with different superscripts within a row differ significantly at $P < 0.001$

applied combined additive (CA) significantly reduced the growth of moulds in silage exposed to air, compared to the other silages. The counts of these microorganisms in our silages did not exceed the maximum thresholds of 3-4 log cfu g^{-1} of fresh matter for good quality silages (McEniry et al. 2006).

CONCLUSIONS

The colonization of the ensiled biomass with LAB was relatively high. The count of harmful microorganisms exceeded the recommended value. A small count of moulds and the absence of yeasts and mycotoxins in alfalfa silage indicate a high mycological status of these feeds. All the tested mycotoxins were below the detection limits for T-2, HT-2, NIV, DON, ZEN, OTA, AF. The additives clearly improved the fermentation profile but did not increase the oxygen stability of silages *vs* control silage. The combined additive (CA) was more effective than the inoculant (I) in alfalfa ensiling.

REFERENCES

Alonso V.A., Pereyra C.M., Keller L.A.M., Dalcero A.M., Rosa C.A.R., Chiacchiera S.M., Cavaglieri L.R. 2013. *Fungi and mycotoxins in silage: An overview*. J. Appl. Microbiol., 115: 637-643. DOI: 10.1111/jam.12178

AOAC 1990. *Official Methods of Analysis*. 15th Ed., Assoc. Offic. Anal. Chem., Arlington, Virginia, USA.

Bauer J., Meyer K. 2006. *Fungal metabolites in silages: Effects on health of food producing animals*. Übers. Tierernährg., 34(1): 27-55. (in German)

Cheli F., Campagnoli A., Dell'Orto V. 2013. *Fungal populations and mycotoxins in silages: From occurrence to analysis*. Anim. Feed Sci. Technol., 183: 1-16. http://dx.doi.org/10.1016/j.anifeedsci.2013.01.013

Danner H., Holzer M., Mayrhuber E., Braun R. 2003. *Acetic acid increases stability of silage under aerobic conditions*. Appl. Environ. Microbiol., 69(1): 562-567. DOI: 10.1128/AEM.69.1.562-567.2003

Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F. 1999. *Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with Lactobacillus buchneri inhibits yeast growth and improves aerobic stability*. J. Appl. Microbiol., 87: 583-594.

Honig H. 1985. *Determination of aerobic deterioration – System Völkenrode*. Landbauforsch. Völkenrode, SH 2: 1-3.

Jatkauskas J., Vrotniakiene V., Ohlsson Ch., Lund B. 2013. *The effects of three silage inoculants on aerobic stability in grass, clover-grass, lucerne and maize silages*. Agric. Food Sci., 22: 137-144.

Juráček M., Bíro D., Gálik B., Šimko M., Gyöngyová M., Michálková J. 2009. *Influence of bacterial-enzyme additive on nutritive value and mycotoxin contamination of horse bean, alfalfa and oat mixture silages. Slovak J. Anim. Sci., 42: 25-29.*

Kaiser E., Weiss K. 2007. *Nitrate content in green forage – Importance for fermentation quality and ensiling technological measures*. Übers. Tierernährg., 35(1): 13-30. (in German)

Kizilsimsek M., Schmidt R.J., Kung L.Jr. 2007. *Effects of a mixture of lactic acid bacteria applied as a freeze-dried or fresh culture on the fermentation of alfalfa silage*. J. Dairy Sci., 90(12): 5698-5705. DOI: 10.3168/jds.2007-0448

Kukier E., Goldsztejn M., Grenda T., Kwiatek K., Wasyl D., Hoszowski A. 2012. *Microbial quality of compound feed used in Poland*. Bull. Vet. Inst. Pulawy, 56: 349-354.

Kung L.Jr., Myers C.L., Neylon J.M., Taylor C.C., Lazartic J., Mills J.A., Whiter A.G. 2004. *The effect of buffered propionic acid-based additives alone or combined with microbial inoculation on the fermentation of high moisture corn and whole-crop barley*. J. Dairy Sci., 87(5): 1310-1316. DOI: 10.3168/jds.S0022-0302(04)73280-4

McEniry J., O'Kiely P., Clipson N.J.W., Forristal P.D., Doyle E.M. 2006. *The microbiological and chemical composition of baled and precision-chop silages on a sample of farms in County Meath*. Irish J. Agric. Food Res., 45(1): 73-83.

Nkosi B.D., Meeske R., Langa T., Thomas R.S. 2011. *Effects of bacterial silage inoculants on whole-crop maize silage fermentation and silage digestibility in rams*. S. Afr. J. Anim. Sci., 41(4): 350-359. http://dx.doi.org/10.4314/sajas.v41i4.5

Pahlow G., Muck R.E., Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F. 2003. *Microbiology of ensiling***.** In: *Silage science and technology*. D.R. Buxton, R.E. Muck, J.H. Harrison (eds.). Agronomy Monograph, 42: 95-139. DOI: 10.2134/agronmonogr42.c3

Pahlow G., Weissbach F. 1999. *New aspects of evaluation and application of silage additives. Contributions of grassland and forage research to the development of systems of sustainable land use*. Landbauforsch. Völkenrode, SH 206: 141-158.

Polan C.E., Stieve D.E., Garrett J.L. 1998. *Protein preservation and ruminal degradation of ensiled forage treated with heat, formic acid, ammonia, or microbial inoculant*. J. Dairy Sci., 81(3): 765-776. http://dx.doi.org/10.3168/jds.S0022-0302(98)75633-4

Purwin C., Fijałkowska M., Lipiński K., Wierzbowska J., Kobzhassarov T.Z., Michalski J. 2015. *Changes in amino acid composition during ensiling lucerne and red clover in round bales*. J. Elem., 20(4): 965-973. DOI: 10.5601/ jelem.2015.20.1.863

Purwin C., Łaniewska-Trokenheim Ł., Warmińska-Radyko I., Tywończuk J. 2006. *Silage quality: Microbiological, health-promoting and production aspects*. Med. Wet., 62(8): 865-869. (in Polish)

Purwin C., Pysera B., Sederevičius A., Makauskas S., Traidaraitė A., Lipiński K. 2010. *Effect of silage made from different plant raw materials with the addition of a fermentation inhibitor on the production results of dairy cows*. Vet. Med. Zoot., 51(73): 44-51. http://vetzoo.lva.lt/data/vols/2010/51/pdf/purwin.pdf

Purwin C., Pysera B., Tokarczuk M., Sederevičius A., Savickis S., Traidaraitė A. 2009. *Production results of dairy cows fed grass and alfalfa silage with a different degree of wilting*. Vet. Med. Zoot., 46(68): 60-66. http://vetzoo.lsmuni.lt/data/vols/2009/46/pdf/purwin.pdf

Rossi F., Dellaglio F. 2007. *Quality of silages from Italian farms as attested by number and identity of microbial indicators*. J. Appl. Microbiol., 103(5): 1707-1715. DOI: 10.1111/j.1365--2672.2007.03416.x

SAS 2009. *User's Guide: Statistics*. Version 9.2. SAS Institute Inc. Cary, NC.

Thaysen J. 2004. *Production of high-quality grass silages*. Übers. Tierernährg., 32(1): 57-102. (in German)

Wagner W., Wolf H., Losand B. 2007. *Evaluation of the microbial quality in silages*. Übers. Tierernährg., 35(1): 93-102. (in German)

Wambacq E., Latré J.P., Haesaert G. 2013. *The effect of Lactobacillus buchneri inoculation on the aerobic stability and fermentation characteristic of alfalfa-ryegrass, red clover and maize silage*. Agric. Food Sci., 22: 127-136.

Weissbach F. 1992. *Determination of the buffer capacity*. Methodenvorschrift am Institut für Grünland – und Futterpflanzenforschung, Bundesforschungsanstalt für Landwirtschaft (FAL), Braunschweig-Völkenrode, 3. (in German)

Weissbach F., Kuhla J. 1995. *Material losses in the determination of the dry matter content of silage and green fodder: resulting error and ways of correcting*. Übers. Tierernährg., 23: 189-214. (in German)

Wilkinson J.M., Davies D.R. 2012. *The aerobic stability of silage: Key findings and recent development*. Grass Forage Sci., 68: 1-19.

Zielińska K., Fabiszewska A., Stefańska I. 2015.*Different aspects of Lactobacillus inoculants on the improvement of quality and safety of alfalfa silage*. Chilean J. Agric. Res., 75(3): 298-306. DOI: 10.4067/S0718-58392015000400005