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Biogenic amines and hygienic quality of lucerne silage

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Abstract: This experiment examined the influence of two different silage additives of biological (*Lactococcus lactis, Lactobacillus plantarum, Enterococcus faecium,* enzyme xylanase) and chemical (43% formic acid, 30% ammonium formate, 10% propionic acid, 2% benzoic acid) types on biogenic amines concentration, nutrient content, fermentation process, and microbiologic indicators in lucerne (*Medicago sativa*) silage after 90 days of fermentation. The biological additive significantly ($P < 0.05$) increased putrescine (+51%), lactic acid (+11%) and protein content (+11%) in comparison with control silage. It significantly decreased cadaverine (–29%), histamine (–57%), spermidine (–15%), spermine (–55%), acetic acid (–40%), ethanol (–55%), ammonium (–25%) and ash (–9%). After the chemical-additive treatment, greater amounts of histamine and tyramine were recorded. Significant decrease was observed in the concentrations of putrescine (–18%), cadaverine (–55%), spermidine (–47%), spermine (–45%), lactic acid (–16%), acetic acid (–46%), ammonium (–59%), ash (–13%) and fat (–24%). Populations of bacteria associated with lactic acid fermentation, moulds, yeasts, enterobacteria and total microorganisms count were also influenced. Both biological and chemical additives can be highly recommended for producing high-quality silages meeting hygienic requirements. In lucerne silage, the chemical preservative showed a stronger effect in achieving the health safety of silage compared to the biological inoculant.

Keywords: health safety, silage additives, microorganisms

1 Introduction

Biogenic amines comprise a group of aliphatic, heterocyclic or aromatic bases derived from amino acids. Biogenic amines are present in all feeds that contain proteins or free amino acids, and they also exist in fermentative feeds [1-3]. The concentration of biogenic amines (mono-, di- and polyamines) in silage and in the rumen, body tissues and body fluids mainly depends upon the crop at harvest, the ensiling process, the silage and the digestion in the animal. Both the synthesis and chemical structures of mono- and diamines are well documented. The basis for their formation is proteolysis, a naturally occurring process in ensiling consisting in the enzymatic decarboxylation of amino acids by the action of plant proteases and peptidases along with enzymes of various lactic acid bacteria (LAB), clostridia and other bacterial genera [4]. High-quality feedstuffs and proper nutrition are important for all categories of livestock [1-3]. High levels of biogenic amines are frequently observed in silages prepared from high-protein forages (lucerne, clover, certain grass species). Some biogenic amines have significant biological characteristics, as they are, for example, tissue hormones (histamine), protoalkaloids (hordenine, gramine) and building

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blocks for biosynthesis of other hormones in animals (phenylethylamine), phytohormones, auxins, alkaloids and other secondary metabolites of plants [5,6]. Histamine, tyramine and 2-phenylethylamine have been found to have negative effects, influencing the nervous system, affecting blood pressure, and provoking skin manifestations. Polyamines (spermidine, putrescine, cadaverine) are essential for cell division and growth, for maintaining high metabolic rate, for immune system function, and for other processes. They also support the growth of tumours, despite their not being carcinogenic on their own [7,8]. The toxic effect of biogenic amines is highly influenced by the activity of these enzymes, which may differ by individual and is dependent on several factors, such as the presence of inhibitors (some medicines, alcohol). When evaluating toxic effect, it is necessary to consider not only the presence of a specific biogenic amine but also other factors such as the amount of a foodstuff consumed and presence of other toxic substances. It is therefore difficult to state the bounds of their toxicity [9]. The use of chemical substances based on organic acids, and particularly those containing larger quantities of formic acid, influence in a positive way the content of nitrogenous substances in lucerne silages. Formic acid positively affects preservation and maintenance of protein content, and especially of those proteins composed from larger numbers of amino acid components [10,11]. The addition of heterofermentative bacteria to promote lactic acid fermentation has been shown to have no effect on nutrient indicators of silages [12,13]. Heterofermentative species of lactobacilli are characterized as having antimicrobial effects, and *Lactobacillus plantarum* has high antimicrobial effects. This finding increases the possibility that a higher density of heterofermentative LAB species with potentially probiotic characteristics would have a positive influence on the resulting quality of preserved feeds [14,15]. High-quality silage depends on the development of favourable microflora under anaerobic conditions. Silage treatments with homofermentative and heterofermentative lactobacilli can achieve considerably better aerobic stability. Additive application of lactobacilli species decreases pH, as well as levels of butyric acid, alcohol and ammonium [16]. The aim of the present study was to determine the influence of two types of treatment (biological and chemical) on the occurrence of biogenic amines, nutrient value, and hygienic quality of lucerne silages. The research hypothesis was that chemical and biological additives would decrease the contents of biogenic amines and unfavourable micro-organisms, thereby improving the hygienic quality of silages.

2 Material and Methods

Experimental material was obtained from Research Institute for Fodder Crops, Ltd. Troubsko (Czech Republic). In the model experiment, lucerne *(Medicago sativa, var. Pálava)* was obtained from the first cutting of an experimental plot. The experimental plot was located in Troubsko. The lucerne was grown in a sugar beet area at an altitude of 270 m. The prevalent soil type there consists of brown soil, and the soil texture is silty to clayey. The experimental parcels had been fertilized with pulverized limestone (10 t ha^{-1}).

Lucerne was harvested in the bud stage while leaving stubble height of 8 cm. The plant material was left to wilt for a short time and then cut to length of 3–4 cm. It was then dried for 6 h to 35% dry matter content. A model experiment was set up whereby the observed factors were the addition of biological and chemical silage additives and their influence on the silage microflora. An experimental control variant (**group K**) was used (with addition of an equivalent amount of potable water in order to maintain the same content of dry matter). The biological silage additive (**group B**), applied at a rate of 20 g/t, consisted of the lactic acid bacteria *Enterococcus faecium (DSM 22502/NCIMB 11181)*, *Lactococcus lactis (NCIMB 30117)*, and *Lactobacillus plantarum (DSM 16568)*, as well as the enzyme xylanase EC 3.2.1.8 in the total amount of 1.25×10^{10} KTJ/g. A chemical preservative (**group CH)** was applied at the rate of 2 L t^{-1}. The chemical additive consisted of 43% formic acid, 30% ammonium formate, 10% propionic acid and 2% benzoic acid. Each variant (K, B, CH) was done in three repetitions, and each experimental silage variant was made in three repetitions. Moreover, the samples were analysed in three repetitions. In total, therefore, 27 silage samples were analysed.

Treated forage material weighing 8 kg was in each case compacted, weighed and anaerobically sealed. The silage density was 750 kg/m^2. The model silages were stored in a laboratory at average temperatures within the range 20–25°C for a period of 3 months. After the silage tubes were opened, they were weighed and from each variant a representative sample was taken in order to evaluate moisture content, fermentation process, nutrient content and biogenic amines, as well as for microbiological analysis.

2.1 Microbiology

Twenty grams of a given sample was shaken in a flask on a Promax 1020 reciprocating platform shaker (Heidolph, Germany) together with 180 mL of physiological solution.

Next, a ten-fold serial dilution was done and 1 mL from the corresponding dilution was inoculated onto a Petri dish and layered with cultivating medium.

Plate count agar (Biokar Diagnostics, France) was used as cultivation medium for determining the total microorganisms count. Inoculation was at 30°C for 72 h. For LAB determination, an MRS agar (i.e. *Lactobacillus* agar in accordance with DeMan, Rogosa and Sharpe; Biokar Diagnostics, France) was used. Inoculation was again for 72 h at 30°C. Chloramphenicol glucose agar (Biokar Diagnostics, France) was used in determining the total count of yeasts and moulds, with incubation lasting 120 h at 25°C. To determine bacteria of the *Enterobacteriaceae* family, violet red bile glucose (VRBG) agar (Biokar Diagnostics, France) was used, with incubation for 24 h at 37°C. After incubation, the grown colonies were read from the Petri dishes and the results were expressed in colony-forming units (CFU) per gram of silage.

2.2 Preparation of water leaching for assessment of fermentation indicators

A sample of silage weighing 50 g was suffused with 450 mL of distilled water. After 24 h the leachate was filtered through filtrating paper (FILTRAK 388) and analysed.

2.3 Nutrient analysis

Ash was determined by weighing the material remaining after burning a fixed-weight sample at 550°C in specified conditions. Fat was determined by weighing the direct extract taken from a sample using petroleum ether on an extraction device according to Soxhlet. Nitrogenous substances were measured by Kjeldal method (N * 6.25 coefficient) using a Kjeltec 2300 device (Foss, Hillerød, Denmark).

Acid detergent fibre (ADF), neutral detergent fibre (NDF), and total fibre were measured on an ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) by two-step hydrolysis in a boiling solution of sulphuric acid. Potassium hydroxide was used in analysing the remaining fibre content, including specification of ash content from the rest of the sample. NDF and ADF contents were determined using detergents sodium lauryl sulphate in the case of NDF and cetyltrimethylammonium bromide in the case of ADF [17].

2.4 Biogenic amines analysis

2.4.1 Preparation of samples

Lucerne samples weighing 300 mg were transferred to a grinding mortar where they were eroded by liquid nitrogen (pouring liquid nitrogen into the grinding mortar with the lucerne and grinding with a pestle for about 2 min). These samples were homogenized with 2 mL of deionised Milli-Q water, then incubated at 25°C and 300 rpm for 60 min on a Thermomixer comfort (Eppendorf). After incubation, the samples were centrifuged at 4°C and 25 000 g for 10 min (Centrifuga 5417R, Eppendorf). After centrifugation, the supernatant was removed and the sample was centrifuged again (4°C, 25 000 g, 10 min). Supernatant was once again removed and the sample prepared in this manner then analysed using liquid chromatography.

2.4.2 Determination of biogenic amines

An AAA 400 ion exchange liquid chromatography apparatus (Ingos, Prague, Czech Republic) was used for determination of biogenic amines. The system consisted of a glass filling chromatographic column and steel pre-column, two chromatographic pumps for transport of elution buffers and derivatization reagent, a cooled carousel for 25 Eppendorf tubes, dosing valve, heat reactor, Vis detector, and cooled chamber for derivatization reagent. The volume of the injected sample was 100 µL, and the application's precision was within 1% relative standard deviation. We used a two-channel Vis detector with a 5 µL flow volume cuvette operated at wavelengths of 440 and 570 nm. A solution of ninhydrin was prepared with 75% methyl cellosolve (v/v) and 25% 4 M acetic buffer (v/v). Tin chloride was used as a reducing reagent. The prepared solution of ninhydrin was stored under an inert atmosphere (N_2) with cooling at 4°C. Flow rate was 0.25 mL min^{-1} under a pressure ranging from 4.5 to 6.0 MPa. Reactor temperature was set to 120°C. For elution, two buffers were employed: buffer A was composed of 5.5 mM $C_6H_8O_7$, 81 mM $Na_3C_6H_5O_7$, 257 mM NaCl, 350 mM KBr, and 250 mL of C_3H_8O per 1 L of Milli-Q water, with a final pH of 5.78. Buffer B consisted of 73 mM $C_6H_8O_7$, 3 M NaCl, and 10.0 mL of 50% solution of KOH (w/w) per 1 L of MilliQ water, with a final pH of 3.27. A WTW inoLab pH meter (Weilheim, Germany) was used for pH measurements.

2.5 Statistics

The data were processed statistically using STATISTICA. CZ, version 10.0 (Czech Republic). Results are expressed as means ± standard deviation. Parametric distribution was analysed by Kolmogorov-Smirnov test. For neparametric data in microbiological analysis the Kruskal-Wallis test was used and for parametric data statistical significance was determined by examining the differences among groups using ANOVA and Scheffé's test (one-way analysis) for the following parameters: histamine, tyramine, putrescin, cadaverine, spermine, spermidine, formic acid, acetic acid, ethanol, ammonium, ash, protein, fat, fibre, ADF, NDF, yeasts, moulds, LAB, and total count of micro-organisms. Differences with $P < 0.05$ were considered significant.

3 Results

Levels of biogenic amines, nutrient composition, amounts of fermenting acids, and microbiological markers in lucerne silage were measured and compared among treatments of the ensiled material using biological or chemical silage additives.

Among biogenic amines, we measured the contents of histamine, tyramine, putrescine, cadaverine, spermidine and spermine (Table 1). Histamine was observed in the B group to be decreased by 57% ($P < 0.05$) in comparison with the control group while in the CH group its content increased by 63% ($P < 0.05$). Tyramine was higher in groups B and CH by 37% and 34%, respectively, relative to the control group. The highest putrescine concentration was in group B, where in comparison with the control group the increase was 51% ($P < 0.05$). In the CH group, a significant 18% decrease of putrescine was observed. Cadaverine contents were significantly lower in groups B and CH by 29% and 55%, respectively. Similar results were observed in levels of spermidine, which were 15% lower ($P < 0.05$) in group B and 47% lower in group CH ($P < 0.05$). There also occurred significant decrease of spermine in both groups B and CH by 55% and 45%, respectively.

Fermentation products measurements are reported in Table 2. pH values ranged from 4.37 to 4.96. With the addition of silage additives, pH decreased by a significant 11.7% in the B group and 11.9% in the CH group. In silages treated with biological additive, the contents of lactic acid, which is beneficial for successful fermentation, increased by 11% ($P < 0.05$) in the B group. After treatment with the chemical additive, lactic acid content decreased by 16% ($P < 0.05$) in comparison with the control group. Acetic acid content was also significantly lower in both experimental groups: by 40% in the B group and 46% in the CH group. Ethanol decreased by 55% ($P < 0.05$) in the B group as compared to the control group. Ethanol concentration in the CH group was under the detection threshold. Ammonia values decreased significantly in comparison with the control group: by 59% in the B group and 25% in the CH group.

Table 3 reports mean nutrient content values. Ash was observed to decrease significantly in the B group by 9% and in the CH group by 13% as compared to the control group. The amount of protein in the B group increased by a significant 11%. The CH group had protein levels

Table 1. Influence of biological and chemical treatments on levels of biogenic amines.

	Biogenic amines (µg/kg)					
Groups	**Histamine**	**Tyramine**	**Putrescine**	**Cadaverine**	**Spermidine**	**Spermine**
Control	13.7 ± 1.0 [a]	10.4 ± 1.2 [a]	33.4 ± 1.2 [a]	9.1 ± 0.3 [a]	15.2 ± 0.5 [a]	50.9 ± 2.2 [a]
Biological (B)	5.9 ± 0.6 [b]	14.2 ± 3.8 [b]	50.9 ± 1.7 [b]	6.4 ± 0.3 [b]	12.9 ± 1.1 [b]	22.7 ± 3.3 [b]
Chemical (CH)	22.3 ± 1.6 [a]	13.9 ± 0.6 [b]	27.6 ± 0.4 [c]	4.1 ± 0.8 [b]	8.1 ± 1.1 [b]	28.0 ± 1.4 [b]

Means ± standard deviation with differing superscripts differ significantly ($P < 0.05$).

Table 2. Influence of biological and chemical treatments on content of fermentation products.

	Fermentation products (g/kg)			
Groups	**Lactic acid**	**Acetic acid**	**Ethanol**	**Ammonia**
Control	113.2 ± 6.0 [a]	27.8 ± 1.3 [a]	9.4 ± 0.3 [a]	2.6 ± 0.1 [a]
Biological (B)	125.9 ± 7.0 [b]	16.6 ± 1.7 [b]	4.3 ± 0.4 [b]	0.4 ± 0.1 [b]
Chemical (CH)	94.9 ± 2.1 [c]	14.9 ± 0.4 [b]	0.0 ± 0.0 [c]	1.1 ± 0.1 [b]

Means ± standard deviation with differing superscripts differ significantly ($P < 0.05$).

practically identical to those for the untreated control group. Fat amount was not significantly affected in the B group, whereas in the CH group a significant 24% decrease in fat was observed. No significant differences were observed among any of the experimental groups in terms of total fibre content, ADF or NDF.

Results from the microbiological analyses are reported in Table 4. The numbers of bacteria of the *Enterobacteriaceae* family were within the order of 10^1 CFU/g for all silage groups. The highest yeast and mould contents reached maximum values on the order of 10^2 CFU/g in all experimental variants. Moulds were decreased in the B group by a significant 73% as compared to the control group. LAB numbers reached values in the order of 10^7 CFU/g of silage in groups K and B. Variants treated with the chemical additive had LAB values one order of magnitude lower. The total number of micro-organisms in experimental silage across all variants was in the range of 10^6–10^7 CFU/g. The total number of micro-organisms includes both desirable and undesirable micro-organisms.

4 Discussion

Because lucerne has a higher protein content in comparison to maize, it is more susceptible to creation of effective biogenic amines. These amines constitute one of the most important risk factors in dairy cattle nutrition. The European Union has not to date established any limits for the amounts of biogenic amines within lucerne silages, and impacts on animals' health are only rarely evaluated. Previously studies have differed in their interpretations as to effects and doses of biogenic amines in farm animals. For histamine, tyramine, putrescine and cadaverine, and depending on the animal category, safe doses of 2–40 g of amine/kg of dry matter have been stated [4]. When using the chemical silage additive Chemisile (2.5 kg/t), Selwet *et al.* observed a decrease of biogenic amines in lucerne silages [18]. Histamine levels in chemically treated versus untreated control silages have been reported as decreasing by 50% while these values were –17% for putrescine, –33% for cadaverine, and –21% for tyramine [19]. A similar effect of chemical treatment was observed in our experiment, wherein the contents of putrescine and cadaverine significantly decreased. The use of two biological silage inoculants (*L. casei* at 10^6 CFU/g and *L. buchneri* at 10^6 CFU/g) in ensiling maize are reported to have had a marked effect on decreasing biogenic amines in maize silage. *L. casei* decreased the occurrence of histamine, tyramine, putrescine and cadaverine. *L. buchenri* reduced only putrescine and histamine [20]. In contrast to these results, we observed the occurrence of histamine to be highest for the biological treatment. Our results suggest the chemical silage additive to be more effective from the perspective of eliminating biogenic amines, leading as it did at the onset of fermentation to a rapid decrease of pH to 4.37 while after treatment with a biological silage additive the pH was 4.96. In another experiment in which inoculant doses were selected similar to those used in our case, a decrease of biogenic amines was observed. At the same time, VanOs *et al.* confirm that unless the

Table 3. Influence of biological and chemical treatments on nutrient content in lucerne silages.

	Nutrients (g/kg)					
Groups	**Ash**	**Protein**	**Fat**	**Fibre**	**ADF**	**NDF**
Control	102.0 ± 4.5 ^{a}	194.6 ± 5.9 ^{a}	49.7 ± 5.4 ^{a}	263.6 ± 11.2 ^{a}	288.9 ± 10.8 ^{a}	349.7 ± 8.1 ^{a}
Biological (B)	92.5 ± 1.6 ^{b}	215.1 ± 4.9 ^{b}	51.8 ± 3.1 ^{a}	270.0 ± 6.5 ^{a}	293.4 ± 11.5 ^{a}	374.9 ± 6.1 ^{a}
Chemical (CH)	88.6 ± 3.8 ^{b}	194.3 ± 2.5 ^{a}	38.0 ± 3.4 ^{b}	254.0 ± 12.7 ^{a}	283.9 ± 13.3 ^{a}	353.8 ± 14.3 ^{a}

ADF: acid detergent fibre, NDF: neutral detergent fibre. Means ± standard deviation with differing superscripts differ significantly ($P < 0.05$).

Table 4. Influence of biological and chemical treatments on microbiological markers.

	Microbiological markers (CFU/g)				
Groups	**Enterobacter**	**Moulds**	**Yeasts**	**LAB**	**TMC**
Control	18.2 ± 4.5 ^{ab}	142.4 ± 10.5 ^{a}	19.7 ± 6.4 ^{a}	$1.6 \times 10^7 \pm 45 \times 10^5$ ^{a}	$1.7 \times 10^7 \pm 65 \times 10^5$ ^{a}
Biological (B)	12.1 ± 1.0 ^{a}	37.9 ± 12.1 ^{b}	57.6 ± 8.3 ^{b}	$1.2 \times 10^7 \pm 54 \times 10^5$ ^{a}	$4.1 \times 10^7 \pm 56 \times 10^5$ ^{a}
Chemical (CH)	36.4 ± 3.2 ^{b}	240.9 ± 45.2 ^{a}	531.8 ± 105.9 ^{b}	$0.5 \times 10^7 \pm 0.7 \times 10^5$ ^{a}	$0.4 \times 10^7 \pm 0.3 \times 10^5$ ^{a}

Enterobacter.: the *Enterobacteriaceae* family, LAB: lactic acid bacteria, TMC total micro-organisms count. Means ± standard deviation with differing superscripts differ significantly ($P < 0.05$).

silage material is acidified within the first 10 days of fermentation, the production of biogenic amines rapidly increases [21].

Jambor [22] also tested the effect of formic acid and the preservative Bactozyme upon the presence of biogenic amines in lucerne haylage where the most abundant biogenic amine (65% of total biogenic amine) was tyramine. In our experiment, this place was held by putrescine (after treating with the biological silage additive). Similar conclusions were also reached by Steidlova *et al.*, [23] who observed that the biological silage inoculant Microsil significantly suppressed the occurrence of tyramine and putrescine.

The hygienic quality of silage is frequently connected with the occurrence of micro-organisms, the activity of which causes forage to heat up, thereby decreasing the contents of nutrients and energy while also producing metabolites (toxins). Holzer *et al.* [24] have stated that undesirable microflora consist of bacteria (including enterobacteria and clostridia), yeasts and moulds. These contribute to reducing the value of forages and subsequently may cause health problems in animals or changes in animal products. Bacteria of the *Enterobacteriaceae* family are frequently considered hygienic markers of faecal pollution [4,25].

In accordance with previously known information, the decrease of biogenic amines after biological and chemical treatment determined in our experiment is due to inhibiting the activity of micro-organisms causing proteolysis connected with the occurrence of biogenic amines [4]. Holzer *et al.* [24] had confirmed the importance of LAB especially in the first phase of fermentation, at which time there occurs a decrease of pH and creation of lactic acid. This acidity has a preservative effect inasmuch as it reduces the development of undesirable micro-organisms (yeasts, moulds, clostridia and enterobacteria). Gallo *et al.* reported observing a higher occurrence of biogenic amines in maize silages with increasing occurrence of clostridia [26]. Bacteria of the *Enterobacteriacea* family are especially important from the hygiene perspective. To date, no acceptable limits for moulds and yeasts exist for the Czech Republic. Although our results indicate that the amount of yeast present was greatest in the experimental silages treated with a chemical silage additive, those yeasts did not create ethanol. This can be the case because the yeasts used the organic acids contained in the chemical silage additive for their metabolism. Considering the low values of yeasts and moulds contained in silages, it can be stated that the silage samples conformed to hygienic requirements for silages. Kaldmäe *et al.* [27] described a positive effect of biological silage additives on the fermentation process. At the same time, they observed a positive influence of biological silage additives on lactic acid values, which were the highest in comparison with the control group. Similar findings were reported by Filya *et al.* [28] and Holzer *et al.* [24]. Hassanat *et al.* [29] report from their experimental work values of ADF, NDF, lactic acid and acetic acid similar to those determined in our experimental lucerne silages, as well as similar absence of propionic acid and butyric acid.

5 Conclusion

This experiment compared two treatments (one biological, one chemical) when ensiling lucerne. The biological treatment decreased the occurrence of some biogenic amines (cadaverine, histamine, spermidine, spermine), fermentation products (acetic acid, ethanol, ammonium) and ash. Microbiological analysis showed there was a decrease in the population of moulds and bacteria of the *Enterobacteriaceae* family. Levels of putrescine and lactic acid increased. Meanwhile, the chemical additive decreased the levels of biogenic amines (putrescine, cadaverine, spermidine, spermine), fermentation products (lactic acid, acetic acid, ammonium), pH, ash and fat. This treatment also reduced total micro-organism counts. Levels of histamine and tyramine were increased. The biological additive supported the fermentation process, and the lucerne silage met the hygienic requirements.

Our results indicate that biological and chemical additives can be recommended for promoting the fermentation process and meeting hygienic standards in producing lucerne silages. Chemical additives are more effective compared to biological silage ones.

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