EFFECT OF BACTERIAL INOCULANT ON MYCOTOXIN CONTAMINATION OF ALFALFA SILAGE

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ABSTRACT

The study aimed to investigate the hygienic quality of alfalfa silages, with a focus on the observed concentrations of mycotoxins. It also examined the effect of biological additives, based on homo- and heterofermentative strains of lactic acid bacteria, on the mycotoxin contamination of alfalfa silages. The study monitored several mycotoxins, including total ochratoxins (OTA), total aflatoxins (AFL), total fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEA), and T-2 toxin (T-2). Mycotoxin concentrations were determined by spectrophotometric immunoenzymatic method (Elisa Reader, Noack SR; Veratox assays, Neogen Ltd., USA) at a wavelength of 650 nm, in alfalfa silage samples that were ensiled into silage units in a volume of 3.5 dm^3. The samples were ensiled in control variant C, without the

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addition of additive, and in experimental variant A with the addition of biological additive (Lactobacillus plantarum, Lactobacillus buchneri) 1.3 x 10^{11} KTJ.g^{-1} at a dose of 10 ml per ton. Both control and experimental treatments were fed in 3 replications (n=3). Analysis of mycotoxic contamination of alfalfa silages revealed statistically significantly ($p < 0.05$) lower fumonisin content in the experimental variant (47.08 μg.kg^{-1}) compared to the control (70.77 μg.kg^{-1}), with a difference of 33.48%. Also, a lower aflatoxin content (3.69 μg.kg^{-1}) was found in the experimental variant compared to the control (4.03 μg.kg^{-1}), which was 8.44%, but these differences were not statistically significant. The contents of DON, ZEA, T-2 and OTA were higher in the experimental variant compared to the control. The mean mycotoxin content of the samples studied showed that alfalfa silages were the most contaminated with ZEA (360.00 μg.kg^{-1}), followed by DON (209.30 μg.kg^{-1}), T-2 toxin (80.38 μg.kg^{-1}), FUM (58.92 μg.kg^{-1}), OTA (53.37 μg.kg^{-1}) and were the least contaminated with AFL (3.81 μg.kg^{-1}). The concentrations of the monitored mycotoxins in alfalfa silages did not exceed the limit values applicable in the EU, which is a prerequisite for ensuring efficient and safe production of animal products.

Keywords: alfalfa silage; mycotoxins; hygienic quality; bacterial inoculant

INTRODUCTION

Silage is the main feed in ruminant nutrition. Silage is a method of preserving forage which allows forage to be stored for a longer period, preserving the nutritional value of the preserved forage. The essence of ensiling is an anaerobic fermentation process in which the pH is reduced by the production of organic acids (mainly lactic acid) by microorganisms, mainly lactic acid bacteria (LAB; Doležal et al., 2012; Mitrík, 2018; Bíro et al., 2020). However, the growth of microscopic filamentous fungi and potential mycotoxin formation can be triggered by factors such as poor compression, inappropriate dry matter content, insufficient hermetic closure, or rainwater infiltration (Rodríguez - Blanco et al., 2021). The growth of microscopic filamentous fungi also occurs after the opening of the silo when the anaerobic stable environment is disturbed, when the pH is increased and thus the preservative effect of organic acids that suppressed their growth is reduced (Kung et al., 2018). Additives based on homo- and heterofermentative lactic acid bacteria (LAB) strains are often used to enhance the ensiling process and improve silage quality. In addition to improving and accelerating the fermentation process, they also improve the aerobic stability of the silage produced. Fermentation acids are fungistatic, and some LAB strains have the potential to reduce mycotoxin content in silages (Kung et al., 2018; Muck et al., 2018; Gallo et al., 2022). For example, Ma et al. (2017) reported that inoculating *L. plantarum or L. buchneri* linearly reduced aflatoxin B_1 (AFB_1) content in silages, but it is not confirmed whether their antifungal and antimycotoxigenic effects can persist up to the silage feeding stage. The growth of microscopic filamentous fungi and their ability to produce mycotoxins in forages is influenced by a complex of biotic and abiotic factors such as species, its aggressiveness. Furthermore, there are environmental factors such as temperature and humidity, or agrotechnical practice (Alonso et al., 2013). The presence of microscopic filamentous fungi in silage does not automatically imply mycotoxic contamination, and vice versa, the absence of filamentous fungi does not guarantee the absence of mycotoxins (Zain, 2011).

There are more than 400 known species of mycotoxins that are commonly found, but only a few are intensively monitored (Fromme et al., 2016). Ensiled forages can be contaminated by microscopic filamentous fungi and their mycotoxins during the pre-harvest phase (genera *Fusarium, Aspergillus, Alternaria*) and/or post-harvest contamination (species of *Aspergillus* and *Penicillium;* Gallo et al., 2015; Alonso et al., 2013). Mycotoxins are secondary metabolites of microscopic filamentous fungi with low molecular weight. The main mycotoxins that contaminate silages are alflatoxins (AFL), fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEA) and ochratoxin (OTA; Alonso et al., 2013). By their presence in silages, they can harm animal health, reduce feed intake, reduce performance, and damage the liver. Some are also carcinogenic, teratogenic, immunosuppressive and cause mortality. All these lead to significant economic losses (Ogunade et al., 2018). AFB_1 can be considered as the most toxic, it is a potent carcinogen, mutagen and teratogen that is produced by the genus *Aspergillus* (Bakirdere et al., 2012). After ingestion of contaminated silage with AFB_1, it is metabolized to alflatoxin M_1 (AFM_1) in the body of dairy cows and can be excreted from the animals through all body fluids, including milk. Therefore, its concentration is monitored in milk with a limit of 0.05 $\mu g.kg^{-1}$ (JECFA, 2001; Commission Regulation (EU) 2023/915). DON, ZEA, FUM are fusarium mycotoxins. DON inhibits proteosynthesis, has immunotoxic and cytotoxic effects. It negatively affects feed intake and consequently production. ZEA is estrogenic, which can cause reproductive disorders in both males and females. FUM are hepatotoxic and immunotoxic. They are also considered as potential carcinogens (Rodrigues, 2014). The most common situation in silages is contamination with several types of mycotoxins simultaneously, which may potentiate their effect on each other or may act synergistically (Cheli et al., 2013). Ruminants are considered to be quite resistant to mycotoxins, due to their rumen microbiota, which is able to partially degrade them into less toxic components. However, ingestion of silage and other feed contaminated with mycotoxins poses a health risk. At the same time, mycotoxins also pose a risk to human health, due to the potential transmission of these toxins through animal products such as milk and meat (Bennett and Klich, 2003; Fink-Gremmels, 2008).

MATERIAL AND METHODS

In cooperation with the university farm SPU s.r.o. Kolíňany farm Oponice, alfalfa (Medicago sativa) from the first mowing at the stage of buttonization was ensilaged. The mass was wilted to a dry matter content of 37% and cut to a theoretical slice length of 20 mm using a self-propelled cutter. The alfalfa was ensiled in two different ways: C (control) and A (experimental variant). In the control variant C, the wilted mass was ensiled without the addition of additives. In experimental variant A, a liquid biological additive based on homo- and heterofermentative lactic acid bacteria (*Lactobacillus plantarum, Lactobacillus buchneri*) 1.3 x 10^{11} KTJ.g^{-1} at a dose of 10 ml per ton was applied to the mass. Alfalfa silage was preserved in three replications (n=3) in the control variant C as well as in the experimental variant A in silage units with a volume of 3.5 dm3. After hermetic sealing, the silage units were stored in the air-conditioned Laboratory of Forage Conservation at the Institute of Nutrition and Genomics at 22 °C. After 8 weeks of storage, the silage units were averaged and sampled. The mycotoxin content of the average samples was determined. Prior to the determination of mycotoxin concentrations, laboratory samples of alfalfa silage were extracted in 70% methanol (for total aflatoxins, total fumonisins, T-2 toxin and zearalenone), in 50% methanol (for total ochratoxins) and in distilled water for deoxynivalenol. A spectrophotometric immunoenzymatic method at a wavelength of 650 nm (Elisa Reader, Noack SR; Veratox assays, Neogen Ltd., USA) was used to determine the mycotoxin content of the samples. Total ochratoxins (OTA), total aflatoxins (AFL), total fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin (T-2) were determined.

The results were statistically evaluated using SPSS 26.0 (IBM) statistical program using one-way ANOVA (mean, standard deviation, minimal and maximal values). Tukey's test and Independent samples T-test at the $p < 0.05$ level were used to evaluate the statistical significance of differences.

RESULTS AND DISCUSSION

Several species of mycotoxins were determined in alfalfa silage samples with and without addition of the biological additive, the concentrations of which are shown in Table 1.

The deoxynivalenol (DON) content was 87.51% ($p < 0.05$) higher in the sample with biological additive than in the control. Fan et al. (2021) observed a positive effect of bacterial inoculant on reducing the occurrence of DON in alfalfa silage samples. Increased DON content in corn silage samples with the inoculant compared to the control was also observed by Kalúzová (2023). The average DON content in the analyzed samples was 209.30 $\mu g.kg^{-1}$. Ogunade et al. (2018) reported an average DON content of 2150.00 µg.kg^{-1} in alfalfa silages. Hodulíková et al. (2016) reported DON content in alfalfa silages from 114.41 to 120.96 µg.kg^{-1} and Juráček et al. (2012) from 365.00 to 379.20 µg.kg^{-1}.

Statistically significant lower content of total fumonisins (FUM) in samples with biological additive compared to C with a difference of 33.48% ($p < 0.05$) was observed. When inoculant was used in maize silage, increased FUM concentrations were observed (Kalúzová 2023; Bakri, 2021; Gallo et al., 2018). The average FUM content in the analyzed samples was 58.92 µg.kg^{-1}. Huerta-Treviño et al. (2016) found an average FUM content of 91.00 µg.kg-1 (fresh alfalfa), Juráček et al. (2014) only 5.40 to 6.27 µg.kg^{-1} (alfalfa silage).

The zearalenone (ZEA) content was lower in the control variant compared to the variant with the addition of the biological additive. The difference between these variants was 11.51 µg.kg^{-1} which was 3.25%. These differences were not statistically significant. Teller et al. (2012) observed lower ZEA concentration in corn silage samples with addition of biological additive compared to control. Among the mycotoxins studied, ZEA occurred at the highest concentration. Its average content in the tested samples was 360.00 µg.kg^{-1}. Huerta-Treviño et al. (2016) reported an average ZEA content of 199.56 µg.kg^{-1} in alfalfa and Ogunade et al. (2018) 533.80 µg.kg^{-1} in alfalfa silages. Adácsi et al. (2022) reported ZEA concentrations lower than 100.00 µg.kg^{-1} in alfalfa silage samples, and Rodríguez-Blanco et al. (2021) did not observe the occurrence of ZEA in alfalfa silage samples.

The T-2 toxin (T-2) content was also lower in the control than in the experimental variant. The difference was 32.33%. Kalúzová et al.

(2022), Wang et al. (2018) and Latorre et al. (2015) confirmed the change in T-2 toxin concentration by using microbial inoculants in maize silages. The average concentration of T-2 toxin in the samples studied was 80.38 $\mu g.kg^{-1}$. Huerta-Treviño et al. (2016) reported a comparable mean T-2/HT-2 concentration in alfalfa silages of 93.71 $\mu g.kg^{-1}$ and Juráček et al. (2014) ranged from 73.30 to 143.50 $\mu g.kg^{-1}$.

The difference in total aflatoxin (AFL) content between the control and experimental treatments was 8.44% and was statistically non-significant. The experimental variant with the addition of inoculant had a lower AFL concentration. Fan et al. (2021) reported a decrease in AFB1 concentration when bacterial inoculants were used in alfalfa silage. Kalúzová et al. (2022) also noted a decrease in AFL concentration following the addition of an inoculant. The average AFL concentration in the analyzed samples was 3.81 $\mu g.kg^{-1}$. A similar mean AFL content in alfalfa silages of 2.77 $\mu g.kg^{-1}$ was also reported by Huerta-Treviño et al. (2016). Rodríguez-Blanco et al. (2021) found an average AFG1 and AFG2 concentration of 2.21 $\mu g.kg^{-1}$ and 0.91 $\mu g.kg^{-1}$, respectively.

Total ochratoxins (OTA) had a lower concentration in the control sample. The difference ($p < 0.05$) between control and experimental samples was 16.56%. Similarly, Kalúzová et al. (2022) reported higher concentration of OTAs after using inoculant in maize silage. The average OTA content in the samples studied was 53.37 $\mu g.kg^{-1}$. Adácsi et al. (2022) reported OTA concentrations in alfalfa silage samples ranging from < 0.50 to 27.57 $\mu g.kg^{-1}$, Juráček et al. (2014) from 13.30 to 13.80 $\mu g.kg^{-1}$, and Huerta-Treviño et al. (2016) reported an average OTA content of 32.74 $\mu g.kg^{-1}$.

When evaluating the mycotoxin content of wilted alfalfa silage, it can be concluded that the biological additive had a positive effect on reducing the content of total fumonisins ($p < 0.05$). A similar trend was observed for total aflatoxins, but the differences were not statistically significant. For the remaining mycotoxins tested, their content was higher in the variant with the addition of the biological additive (DON, T-2, OTA, ZEA). ZEA (360.00 $\mu g.kg^{-1}$) was the most abundant mycotoxin in the alfalfa silage samples, followed by DON (209.30 $\mu g.kg^{-1}$) and T-2 toxin (80.38 $\mu g.kg^{-1}$). The monitored samples did not exceed the permitted acceptable and recommended limits (Directive 2002/32/EC; Commission Recommendation 2006/576/EC; EFSA, 2014).

Table 1. Average concentrations of mycotoxins in alfalfa silage

µg.kg-1 of DM	**DON**	**FUM**	**ZEA**	**T-2**	**AFL**	**OTA**
Control (C)	145.59^{a}	70.77^{a}	354.24	69.19^{a}	4.03	49.29^{a}
Additive (A)	273.00^{a}	47.08^{a}	365.75	91.56^{a}	3.69	57.45^{a}
Average	209.30	58.92	360.00	80.38	3.81	53.37

DON: deoxynivalenol, FUM: total fumonisins, ZEA: zearalenone, T-2: T-2 toxin, AFL: total aflatoxins, OTA: total ochratoxins. Values with identical indexes in a column are statistically significant ($p < 0.05$).

Previous studies have already indicated that some LAB strains can degrade or inhibit mycotoxins during the fermentation process (Wambacq et al., 2018; Ferrero et al., 2019). Antifungal compounds such as organic acids, carboxylic acids and phenolic compounds produced by LAB can reduce mycotoxins produced by filamentous microscopic fungi (Peles et al., 2019). Piotrowska (2014) confirmed that *Lactobacillus brevis* and *Lactobacillus plantarum* reduced the concentration of OTA in vitro. Franco et al. (2011) in a similar study found that *Lactobacillus brevis* and *Lactobacillus paracas*ei reduced the concentration of DON in vitro. Ma et al. (2017) revealed the capacity of LAB to bind AFB_1 in vitro, but also in corn silage samples artificially contaminated with AFB_1, when the concentration of AFB_1 in the samples decreased. It can be concluded that microbial additives can be used to prevent the growth of filamentous microscopic fungi and to reduce the mycotoxin content.

CONCLUSION

The results confirmed that a biological additive based on homo- and heterofermentative strains of lactic acid bacteria had a positive effect on the demonstrable reduction of total fumonisins. There was also a reduction in the concentration of total aflatoxins, but the differences were not statistically significant. However, an increase in the content of deoxynivalenol (DON), T-2, ochratoxins (OTA), and zearalenone (ZEA) was observed. These findings suggest that further experiments are needed to verify the effect of additives on mycotoxin concentrations in alfalfa silages. Alfalfa silages were the most contaminated with zearalenone, followed by deoxynivalenol and T-2 toxin. The monitored concentrations of all mycotoxins in lucerne silages did not exceed the maximum permitted limits for mycotoxins in ruminant feed, a prerequisite for ensuring the sustainable and safe production of animal products.

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