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The effect of lactic acid bacteria addition on microbiota and occurrence of mycotoxins in rye silages

Vplyv prídavku baktérií mliečneho kvasenia na mikrobiotu a výskyt mykotoxínov v ražných silážach

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ABSTRACT

In the rye silages (control and with additive) microbiota isolation and identification (total count of bacteria, coliform bacteria, enterococci, lactic acid bacteria and microscopic filamentous fungi) and occurrence of mycotoxins were evaluated. Total count of bacteria on Plate count agar at 30 °C for 48-72 hours, coliform bacteria on McConkey agar at 37 °C for 24-48 hours, enterococci on Enterococcus selective agar at 37 °C for 48-72 hours, lactic acid bacteria on De Man, Rogosa and Sharpe agar, Mayeux, Sandine and Elliker and All Purpose TWEEN® agar 37 °C for 48-72 hours in microaerophilic condition and microscopic filamentous fungi on Malt extract agar at 25 °C for 7 days were evaluated and identified with mass spectrometry. The most isolated family from both types of silage was *Lactobacillaceae*. The most isolated species from the control group was *Lentilactobacillus buchneri* (22%) and lowest number of isolated species was *Mucor* spp. The most isolated species from silage with additive was *Lactobacillus gasseri* (16%). The ELISA method (enzyme-linked immunosorbent assay) with using reader at wavelength 650 nm for detection and quantification of mycotoxins (total aflatoxins, total ochratoxins, total fumonisins, deoxynivalenol, zearalenone and T-2/HT-2 toxin) was used. The rye silages of control and with additive were characterized by occurrence of all analyzed mycotoxins, whereas deoxynivalenol was mycotoxin with the highest concentration. The rye silages with additive had significantly lower content of total aflatoxins compared to the control, while significantly higher concentration of total ochratoxins, deoxynivalenol, zearalenone and T-2/HT-2 toxin was determined.

Keywords: biological additive, silage, mycotoxin contamination, bacteria, microscopic filamentous fungi, mass spectrometry

ABSTRAKT

V ražných silážach (kontrolných aj s aditívom) bola hodnotená izolácia a identifikácia mikrobioty (celkový počet baktérií, koliformných baktérií, enterokokov, baktérií mliečneho kvasenia a mikroskopických vláknitých húb) a výskyt mykotoxínov. Pomocou hmotnostnej spektrometrie bol hodnotený a identifikovaný celkový počet baktérií na Plate count

agare pri teplote 30 °C počas 48-72 hodín, koliformných baktérií na McConkeyho agare pri teplote 37 °C počas 24-48 hodín, enterokokov na Enterococcus selektívnom agare pri teplote 37 °C počas 48-72 hodín, baktérií mliečneho kvasenia na De Man, Rogosa a Sharpe agare, Mayeux, Sandine a Elliker a All Purpose TWEEN® agare pri 37 °C počas 48-72 hodín v mikroaerofilných podmienkach a mikroskopické vláknité huby na Sladinovom agare pri 25 °C počas 7 dní. Najviac izolovanou čeľaďou z oboch typov siláže bola *Lactobacillaceae*. Najviac izolovaným druhom z kontrolnej skupiny bol *Lentilactobacillus buchneri* (22%) a najnižší počet z izolovaných druhov mal *Mucor* spp. Najviac izolovaným druhom zo siláže s aditívami bol *Lactobacillus gasseri* (16%). Na detekciu a kvantifikáciu mykotoxínov (celkové aflatoxíny, celkové ochratoxíny, celkové fumonizíny, deoxynivalenol, zearalenon a T-2/HT-2 toxín) sa použila ELISA metóda (enzyme-linked immunosorbent assay) s využitím čítačky pri vlnovej dĺžke 650 nm. Ražné siláže kontroly a s prídavkom aditíva sa vyznačovali výskytom všetkých analyzovaných mykotoxínov, pričom deoxynivalenol bol mykotoxín s najvyššou koncentráciou. Ražné siláže s aditívom mali výrazne nižší obsah celkových aflatoxínov v porovnaní s kontrolou, pričom bola stanovená výrazne vyššia koncentrácia celkových ochratoxínov, deoxynivalenolu, zearalenonu a T-2/HT-2 toxínu.

Kľúčové slová: biologické aditívum, siláž, mykotoxická kontaminácia, baktérie, vláknité mikroskopické huby, hmotnostná spektrometria

INTRODUCTION

Several fungi species of the genera *Fusarium*, *Penicillium* and *Aspergillus* are major producers of mycotoxins. Among the most important mycotoxins are *Fusarium* mycotoxins: fumonisins, deoxynivalenol, zearalenone, T-2 toxin, *Penicillium* and *Aspergillus* mycotoxins: ochratoxins, and *Aspergillus* mycotoxins: aflatoxins. Synthesis of mycotoxins affects many factors such as natural microbiota, interactions between the species and strains, locality, type of feed/silage, storage time, temperature during fermentation, pH value, moisture, accessibility of oxygen, additives and other (Skladanka et al., 2017; Ogunade et al., 2018; Wang et al., 2018; Sadiq et al., 2019). Addition of lactic acid bacteria (LAB) can reduce the concentration of mycotoxins due to the direct inhibition of fungi as their producers (*Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *L. reuteri*, *Lentilactobacillus buchneri*, *L. hilgardii*, *Lacticaseibacillus rhamnosus*, *Lactococcus lactis*, *Pediococcus pentosaceus*, and some strains of *Leuconostoc* genera) (Zielińska and Fabiszewska, 2017; Gallo et al., 2018; Ferrero et al., 2019). Further, some LAB strains can reduce the mycotoxins through adsorption on cell walls, biodegradation, and interactions of metabolites (Ma et al., 2017; Muhialdin et al., 2020). Many studies have confirmed the potential of LAB in mycotoxins reduction in feed and food. Møller et al. (2021) confirmed the ability of LAB to bind zearalenone, ochratoxin A, and aflatoxin B1 what resulted in their reduction at least 50%. Furthermore, the positive impact of *L. plantarum* and *Levilactobacillus* spp. on the inhibition of *Aspergillus parasiticus* and the reduction of aflatoxin B1 was reported. *Lacticaseibacillus paracasei* and *Lactobacillus lactis* degraded zearalenone by 55% (Rogowska et al., 2019), *L. rhamnosus* reduced ochratoxin A by 97% (Luz et al., 2018), *L. plantarum* degraded aflatoxin B1 by 60% (Huang et al., 2017), and deoxynivalenol in the range 56-66% (Franco et al., 2011). According to Nyamete et al. (2016) fumonisin B1 reduction by LAB cultures (*L. plantarum*, *L. fermentum*, *L. casei*, and *Pediococcus pentosaceus*) ranged between 14-30%. Study of Zhou et al. (2017) confirmed degradation of T-2 toxin by *L. lactis* in removal rates from 23.45 to 54.08%. However, the efficiency of LAB in the mycotoxin reduction of feed and food is variable, due to many factors (Perczak et al., 2018; Sadiq et al., 2019; Ragoubi et al., 2021).

The objective of our study was to determine the microbiota and mycotoxins occurrence in the rye silages with the addition of biological additive.

MATERIAL AND METHODS

Silage production

The rye variety Borfuro (*Secale cereale*, L.) was harvested at the flowering growth stage, wilted (48 hours), and then chopped to a 2 cm theoretical cut with the self-propelled harvester (Class Jaguar 850). Wilted

matter (with dry matter of 35%) was ensiled in the control (CO) without additive and inoculated with the additive (AD) on the base lactic acid bacteria (LAB): *Lactococcus lactis*, *Lacticaseibacillus paracasei*, and *Pediococcus acidilactici*; in liquid application form 25 ml/t (with 2 g of lyophilized LAB with $1.25x10^{11}$ CFU/g active bacteria). From each CO and AD three mini-silo bags (using MSW Motor Technics vacuum pack device) were prepared and opened after the ensiling time of 1.5 year.

Microbiota isolation and identification

In the primary dilution of silage, 0.87% sterile saline with the quantity of 45 mL was used to which 5 g of sample was added. Subsequently, serial dilutions (10^{-2} to 10^{-4}) were prepared, and 100 µL of them were applied to PCA agar plates (Sigma-Aldrich®, St. Louis, USA) to determine the total number of bacteria. The presence of bacterial colonies was examined in the inoculated plates after the incubation period of 48-72 h at 30 °C.

Typical colonies of coliforms bacteria were enumerated after 24-48 h (37 °C) of incubation on inoculated McConkey agar (MC, Sigma-Aldrich®, St. Louis, USA) plates. Formation of typical colonies for enterococci was examined with the use of Enterococcus selective agar (ESA, Sigma-Aldrich®, St. Louis, USA), whereas incubation time and temperature were same as for coliforms bacteria. Lactic acid bacteria were cultivated with the use of three different agars, specifically MRS (De Man, Rogosa and Sharpe agar), MSE (Mayeux, Sandine and Elliker) and APT (All Purpose TWEEN® agar, Sigma-Aldrich®, St. Louis, USA). Inoculated plates were incubated under the anaerobic conditions for 72 h at 37 °C. For microscopic fungi and yeasts identification, Malt extract agar (Sigma-Aldrich®, St. Louis, USA) and acid base indicator bromocresol green (Sigma-Aldrich®, St. Louis, USA) (0.020 g/l[1]) were used. The growth on inoculated plates was evaluated after 5 days of aerobic exposure and incubation temperature 25 °C. Because of the macroscopic morphological differences between the growing colonies, recultivation on TSA (Tryptic Soya agar, Oxoid®) was done. The cultivation of inoculated plates took place for 24 h at 30 °C or 25 °C for bacteria and yeasts, respectively. After the cultivation, the proteins extraction was done. Further confirmation of microorganisms (the colonies from total microbial count, coliforms bacteria, enterococci, lactic acid bacteria, fungi and yeasts) was performed using MALDI-TOF. Identification of selected colonies was examined after aerobic or anaerobic subculture on TSA agar overnight. The preparation of microbial isolates for MALDI-TOF MS analysis was previously published by Kačániová et al. (2019) and realized according to the manufacturer´s extraction procedure (Bruker Daltonik, Bremen, Germany). Also, Singh et al. (2017) published identification for fungal isolates. Identification was done by MALDI-TOF MS Biotypes (Bruker Daltonics, Germany) with Flex Control 3.4 software and Biotyper Realtime Classification 3.1 with BC specific software (Bruker Daltonics, Germany).

Mycotoxin analysis

The concentration of mycotoxins (deoxynivalenol: DON, zearalenone: ZEA, T-2/HT-2 toxin: T-2/HT-2, total fumonisins: FUM, total ochratoxins: OTA, and total aflatoxins: AFL) was examined by immunochemical analysis using the Veratox tests by ELISA reader at 650 nm. Before analyzes, extracts of silages were prepared in distilled water (DON), 50% methanol (T-2/HT-2 toxin and OTA), and 70% methanol (AFL, FUM, and ZEA). The results of mycotoxins were subsequently calculated to the 12% moisture content. Dry matter content of silages was measured using the drying method at 103±2 °C. The Oneway ANOVA-descriptive statistics and differences between the treatment and control by Independent Samples T-Test were expressed.

RESULTS AND DISCUSSION

Microbiota

In the control samples ranged lactic acid bacteria from 1.51 log cfu/g on MSE to 3.17 log cfu/g on MRS, number of coliforms bacteria and enterococci were under the detection limit, total number of microorganisms was 3.45 log cfu/g and microscopic filamentous fungi was 2.55 log cfu/g. In the silage with the addition of additive,

lactic acid bacteria ranged from 2.00 log cfu/g on MSE to 4.11 log cfu/g on MRS, number of coliforms bacteria and enterococci were the detection limit, total count of microorganisms was 3.38 log cfu/g and microscopic filamentous fungi was 1.15 log cfu/g (Table 1). The essential part of a silage fermentation process are lactic acid bacteria from the autochthonous microflora (Pahlow et al., 2003). Pahlow et al. (2003) further state, that this group of bacteria has one of the most varying numbers in crops. While alfalfa forage has a detection limit of 10^1 to 10^5 cfu/g, perennial grasses (10^6), corn and sorghum (10^7) have a higher limit of detection. The minimum amount of LAB (5 log cfu/g) that can ensure the good quality of silage fermentation, was firstly recommended by Muck (1991). This is the amount needed to stabilize the silage process through a sufficient production of acids. The most isolated genera from both types of silage was *Lentilactobacillus*. The most isolated species from control group was *Lentilactobacillus buchneri* (22%) and the lowest number of isolated species was *Mucor* spp. (Figure 1). In a study by Schmidt et al. (2008), inoculated silages were higher in numbers of *L. buchneri*, but a different type of determination was used (real-time PCR). The most isolated species from silage with additive was *Lactobacillus gasseri* (16%) (Figure 2). Considering the air present between plant particles during early fermentation stage, aerobic microorganisms like yeasts, moulds and aerobic bacteria grow together with various species of the *Lactococcus* and *Lactobacillus* genera. This plant respiration process gradually becomes anaerobic, with most of the population being LAB (Ohmomo et al., 2002).

Mycotoxins

The all samples of rye silages were contaminated with all determined mycotoxins. DON was the mycotoxin with the highest concentration in the rye silages (Table 2), regardless of the addition of additive. Lower average DON value was observed in the samples of corn silages from Belgium (average 670.00 µg/kg of DM) (Vandicke et al., 2021), whereas Weaver et al. (2021) found out higher DON values (1870.00±2440.00 µg/kg of DM) from the USA, and Del Palacio et al. (2016) in wheat silages (6007.00±2122.00) from Uruguay, compared to the recent study. After the addition of the biological additive, the DON concentration in the rye silages increased ($P<0.05$), which is consistent with the results of Gallo et al. (2021) in the corn silages (control 2215.00 µg/kg of DM vs. LAB 2563.00-4373.00 µg/kg of DM). ZEA was the mycotoxin with the second highest concentration in rye silages. Weaver et al. (2021) reported a lower values of ZEA in comparison with our study (560.00±683.00 µg/kg of DM) in the corn silages, but with the maximal concentration 4021.00 µg/kg of DM. Treatment with additive (AD) resulted in higher ZEA value ($P<0.05$) in rye silages. Differently, Møller et al. (2021) determined the positive effect of *L. plantarum* on reducing ZEA *in vitro*, and confirmed that levels of bind efficiency are influenced by the applied conditions (time of treatment and pH value). The results of T-2/HT-2 are comparable with the findings which reported Weaver et al. (2021) in the corn silages from the USA in samples from 2013-2019 (T-2 11.70±19.6; HT-2 247.00±547.00 µg/kg of DM). The rye silages with the biological additive had higher T-2/HT2 toxin content ($P<0.05$). Differently, Zhou et al. (2017) confirmed degradation of T-2 toxin in the range from 23.45 to 53.17% by different cellular structures of *Lactococcus lactis*. Despite the fact that *L. lactis* was part of the applied additive in the present study, the decrease in T-2/HT-2 was not observed. That could have been due to the fact that *L. lactis* was added with other strains of LAB. Weaver et al. (2021) analysed higher concentration of FUM in the corn silages from the USA (2227.00±5100.00 µg/kg of DM) and otherwise lower (82.00 µg/kg of DM) in investigation by Vandicke et al. (2021) from Belgium than recent study. The value of FUM increased in the rye silages inoculated with LAB, albeit non-significantly ($P>0.05$). Increase of LAB population after the silage additive application probably acts as a stressful factor on *Fusarium* spp. fungi, which resulted in increased concentrations of *Fusarium* mycotoxins. This is consistent with the results of Gallo et al. (2018; 2021) and Saylor et al. (2020). The OTA content was higher ($P<0.05$) in rye silages with additive than in control.

Table 1. The average content of Microbiota in rye silages (log cfu/g)

	MSE	MRS	APT	MC	EA	PCA	MEA
CO	1.51±0.04	3.17±0.05	1.80±0.20	0.00±0.00	0.00±0.00	3.45±0.09	2.55±0.08
AD	2.00±0.10	4.11±0.04	2.32±0.09	0.00±0.00	0.00±0.00	3.38±0.04	1.15±0.06

CO - control, AD - additive, MSE - Mayeux, Sandine and Elliker, MRS - De Man, Rogosa and Sharpe agar, APT - All Purpose TWEEN® agar, MC - McConkey agar, EA - Enterococcus selective agar, PCA - plate count agar, MEA - malt extract agar

Figure 1. Krona chart of isolated microorganisms from control silages

Figure 2. Krona chart of isolated microorganisms from silages with additive

Average OTA concentrations in CO and AD were lower compared to the data of Glamočić et al. (2019) in the corn silages from different regions of Serbia (10.40 µg/kg of DM). Wang et al. (2018) found the average level of OTA in corn silages from China 34.40 µg/kg of DM and confirmed the effect on OTA caused by *L. plantarum* and *Pediococcus pentosaceus* depending on the conservation temperature. Identically determined, that application of *P. pentosaceus* increased the OTA concentration ($P<0.05$) in corn silages stored at 28 °C and differently determined, that addition of *P. pentosaceus* and *L. plantarum* decreased the OTA concentration at storage temperature 37 °C. AFL was the mycotoxin with the lowest concentration with all the determined mycotoxins in rye silages. Lower AFL value was observed (average 3.00 µg/kg of DM) by Schmidt et al. (2015), whereas Keller et al. (2013) found out higher AFL value (average 33.00 µg/kg of DM) in corn silages from Brazil, than current study. The rye silages with the addition of inoculant had statistically significant ($P<0.05$) lower content of total aflatoxins by 16.75%. This was also confirmed by the results of Gallo et al. (2021) in the corn silages from Italy, who recorded the

Table 2. The average concentrations of mycotoxins in rye silages

Mycotoxins in µ/kg of dry matter	Control /CO/		Additive /AD/	
	AVG±S.D.	min.-max.	AVG±S.D.	min.-max.
DON	1341.75±260.43*	1089.23-1627.35	1745.95±129.98*	1628.64-1863.26
ZEA	653.20±15.68*	634.34-668.11	1191.96±46.44*	1143.60-1236.36
T-2/HT-2	207.74±4.07*	203.12-212.85	222.80±9.16*	213.59-231.15
FUM	193.42±15.72	178.87-209.06	221.51±19.85	204.15-243.38
OTA	20.19±0.49*	19.66-20.76	60.42±1.09*	59.49-61.54
AFL	13.49±0.07*	13.43-13.57	11.23±0.50*	10.60-11.83

CO - without additive, AD - *Lactococcus lactis*, *Lacticaseibacillus paracasei*, *Pediococcus acidilactici*; DON - deoxynivalenol, ZEA - zearalenone, T-2/HT-2 - T-2/HT-2 toxin, FUM - total fumonisins, OTA - total ochratoxins, AFL - total aflatoxins, *means with the same superscript within the same row are significantly different at (P<0.05)

concentration of AFB1 22.96 µg/kg of DM in the control and in the silages with the addition of *L. plantarum* 7.85 and *L. rhamnosus* 10.89 µg/kg of DM. The decrease in concentration is related to the ability of some LAB strains to the partially degrade AFLB1 up to 46.00% (Oluwafemi et al., 2010) and can bind to Aflatoxin B1 (Ma et al., 2017). The values of determined mycotoxins did not exceed EU recommendations in complete feedstuffs for adult ruminants except for ZEA (Commission Directive 2003/100/EC; Commission Recommendation 2006/576/EC; Commission Recommendation 2013/165/EU). All samples of rye silages had higher concentration of ZEA as 500 µg/kg of DM representing limit value for complementary and complete feedstuffs for dairy cattle.

CONCLUSION

Total nine species of microorganisms from silages treated by lactic acid bacteria, and eight species of microorganism from untreated silages were isolated. Compared to the control group, more species of *Lactobacillaceae* family were isolated in the treated silage. Therefore, addition of lactic acid bacteria to a silage had a positive effect both for the total groups of microorganism and individual isolated species. The all samples of rye silages were contaminated with all the determined mycotoxins with the highest concentration of DON, followed by the ZEA and with the lowest concentration of AFL. All samples of rye silages were under the limits of EU recommendations except for the ZEA. Addition of lactic acid bacteria as silage additives positively decrease concentration of the total aflatoxins produced by the *Aspergillus* spp. compared to the untreated silages however, increase total ochratoxins and concentrations of mycotoxins produced by *Fusarium* spp. (deoxynivalenol, zearalenone, T-2/HT-2 toxin, total fumonisins).

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