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Effect of lactic acid bacteria inoculation on the aflatoxin B1 contamination and the diversity of yeast communities in *Aspergillus flavus*-contaminated experimental corn silage

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ABSTRACT

The present work aimed to study the yeast communities of whole crop corn silages (CS) that were previously contaminated with aflatoxin-producing *Aspergillus flavus* (CSCA). In addition, the effect of lactic acid bacterium (LAB) inoculation on the aflatoxin B1 (AFB1) content, genotoxicity, yeast load, and diversity of yeast communities were also investigated. In *A. flavus* contaminated silages, after two months, the AFB1 content was 40% lower with LAB inoculation, also a lower level of genotoxicity was determined.

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The number of yeasts cultured from the initial mixture of chopped whole crop corn was 4.8×10^7 CFU g^{-1} wet mass, while only 2.4×10^6 CFU g^{-1} from the CSCA and 7.1×10^5 CFU g^{-1} from the LAB-inoculated CSCA could be cultured. Based on 144 randomly isolated strains, the yeast community of the initial mixture consisted of 8 species. In contrast, the yeast community of CSCA consisted only of 4 species determined by 132 randomly selected isolates. LAB-inoculated CSCA consisted also of 4 species based on 158 randomly isolated strains. *Saccharomyces cerevisiae* and *Pichia kudriavzevii* proved to be predominant in the CSCA, while *S. cerevisiae* and *Meyerozyma guilliermondii* were the most abundant in the LAB-inoculated CSCA. The species richness was also confirmed by alpha diversity values (1.827, 1.188, and 1.123 as Shannon's indices for CS, CSCA, and LAB-inoculated CSCA, respectively). In response to LAB inoculation, the species diversity decreased considerably.

KEYWORDS

yeasts, corn silage, *Aspergillus flavus*, lactic acid bacteria, diversity values

1. INTRODUCTION

Aflatoxins are the most dangerous mycotoxins that are produced by the mould *Aspergillus flavus* and its close relatives, which in addition to their direct toxicity have significant immunosuppressive and carcinogenic effects (WHO-IARC, 2012). Aflatoxins are difuranocoumarin derivatives, i.e., compounds consisting of a linkage of two furan rings (Bennett and Klich, 2003). Their quantitative chemical analysis can be done with HPLC, and their genotoxic effects can be monitored by the semi-quantitative SOS Chromotest. The latter method uses *Escherichia coli* PQ37, which is a genetically modified strain to detect the molecules with genotoxic and cytotoxic abilities, such as aflatoxin B1 (AFB1) mycotoxin. It is known from the literature (Walker, 1987; Legault and Blaise, 1994) that AFB1 induces SOS repair mechanism in bacteria, due to a fusion of two operons; *lacZ*, which is a structural gene for ß-galactosidase taken under the control of *sfiA* gene, which is responsible for the SOS mechanism that repairs damage done to the genetic stock. These two genes play roles in cell division, along with 17 other genes, which leads to the SOS error-prone response in the test organism. Increased amount of the genotoxic compound in the test environment leads to increasing ß-galactosidase production, which can be detected by providing appropriate substrate to the system. Enzyme activity can be detected with colorimetric methods. The test uses S9 rat liver homogenate to examine compounds with genotoxic activity and metabolic activation to display their genotoxic effect.

The most dangerous and best-known member of the aflatoxins is the chemically stable AFB1, which can be transferred to animal meat and milk through contaminated feed. The most relevant source of aflatoxin contamination in milk is silage, the chemical and microbiological status of which depends on a number of factors.

The silages are the worldwide most common fodder for cattle, sheep, and other ruminants. They have been used since the ancient times, but spread in Europe only with the development of large-scale livestock farming (Woolford, 1994). Notable benefit of the ensiling method lies in having low energy demand, saving original moisture of total mixed ration, and nutritive values. Besides, the silage has got a good palatable character and it may improve the rumen degradability of starch-protein matrix, cellulose, and other carbohydrates (Hoffman et al., 2011;

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Carvalho et al., 2017). The main process of the development of silage is performed by an anaerobic fermentation catalysed by different microorganisms, and an outstanding role is played by the populations of lactic acid bacteria (LAB) among them (Ávila and Carvalho, 2019). For the purpose of removing air from the silages, the chopped moist crops are stuffed into silo containers or bales. The speedy growth of the populations of LAB results in a desirable acidification of the silage for sufficient inhibition of the aerobic rotting microorganisms, like aerobic spore-forming bacteria, *Clostridium* species, *Enterobacteriaceae*, yeasts and moulds, and a lot of other harmful rotting microbes (Ogunade et al., 2017). Several residual epiphytic microbial populations can be found in the ensiling substrate, but after a few days, LAB and propionic acid bacteria become predominant along the succession (Liu et al., 2019).

On the base of their fermentation product, three groups of LAB are established: (1) homofermentative LAB, almost exclusively producing lactic acid; (2) obligately heterofermentative LAB, producing different fermentation compounds beside the lactic acid, such as acetic acid, CO_2, and ethanol; (3) LAB belonging to the group of facultatively heterofermentative ones with an intermediate metabolism, especially in view of their metabolic pathways (Axelsson, 2004; Madigan et al., 2015). The most important LAB species living in silage belong to the genera *Lactobacillus, Streptococcus, Pediococcus, Bifidobacterium,* etc., but the most important species belong to the genus *Lactobacillus*. The latter organisms are Gram-positive, non-spore-forming, rod-shaped bacteria, which are homofermentative and dissimilate sugars exclusively through the Embden–Meyerhof pathway (Muck, 2013). LAB represent a low proportion of the epiphytic microorganisms, for this reason, desirable activity of them in the beginning phase of the ensiling process can be increased by the use of inoculants. Research on inoculants for silages significantly progressed in the last decades, big differences were found between strains for growing rate, ability of fermentation, and other physiological features (Muck et al., 2018; Parra et al., 2021). The most commercially available inoculants contain strains belonging to the species *Lactobacillus plantarum, Lactobacillus acidophilus* (Li and Nishino, 2011), *Enterococcus faecium*, and to the genus *Pediococcus* (Porto et al., 2017).

The presence and activity of propionic acid bacteria (PAB) are also very important, because they produce fungistatic compounds like propionic acid, acetic acid, CO_2, and succinic acid; the gaseous CO_2, extending in the matrix, contributes to the anaerobic condition. PAB are Gram-positive, non-spore-forming, rod-shaped or pleomorphic, anaerobic or facultatively anaerobic bacteria that dissimilate sugars through the Embden-Meyerhof pathway (Thierry et al., 2011).

Yeasts, from phylogenetic point of view, form a totally heterogenous fungal group, both the ascomycetous and basidiomycetous taxons can be found among them (Kurtzman et al., 2011). They proliferate by budding or fission, enabling just a slow growth in nutritive liquids even in moderate acidic media (Péter, 2022). The epiphytic, in majority obligate aerobic, yeast population shows a very weak initial growth, but the acidification and the increasing CO_2 concentration totally inhibit them (Lachance, 2006). The permanent yeast population is composed of species that can tolerate the increasing anaerobiosis, the acidic environment, and the varying temperature (Pahlow et al., 2003; Borreani et al., 2018). The most frequently isolated species is *Issatchenkia orientalis* (syn. *Pichia kudriavzevii*), that can use lactic acid as sole carbon source and can alter the ruminal fermentation (Santos et al., 2017). A lot of species belonging, in majority, to the genera *Candida* and *Pichia* also were cultivated from silage as their verified habitat. In addition, the presence of dozens of other yeast species was pointed out by the

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methods of next generation sequencing and DGGE (Denaturing Gradient Gel Electrophoresis) (Keshri et al., 2018; Liu et al., 2019). It should be noted that the activity of the above-mentioned yeasts particularly grows exposed to oxygen in case of technological irregularity or during feed-out in the silage. In the aerobic environment these species begin to assimilate lactate, causing the increasing of pH-value and establishing opportunity for different aerobic microbes, e.g., spore-forming bacteria and moulds. The latter organisms are responsible for the loss of dry matter content in the silage and enhancing the mycotoxin content of the fodder. In some cases, the presence and role of yeasts can be useful in silages (Vohra et al., 2016). Yeasts belonging to the species *Saccharomyces cerevisiae* are often used as probiotics for ruminants, for improving the rumen functions. Although Dunière et al. (2015) suggested that yeast inoculation is a good strategy to introduce direct-fed microbials to the silage production technologies, yet only LAB strains are commercially available.

Some moulds and yeasts are natural members of the epiphytic microbiota of corn, particularly in the second part of the vegetation period (Lindow and Brandl, 2003). In case of the failure of technological regularities, the epiphytic moulds (*Alternaria, Aureobasidium, Cladosporium,* etc.) can survive in the ensiling mixture. Due to their amensalistic effect, the development of LAB population undergoes a considerable delay, in addition, due to their lactate-assimilating ability, a significant decrease in the desirable acidity is resulted (Ávila and Carvalho, 2019). The members of the other ecotype of the moulds in silage (*Aspergillus, Penicillium, Fusarium,* etc.) originate from the spermosphere of the crop plants or arrive as ubiquitous contaminants from the environment to the silage, and can grow there only in case of reduced acidity and imperfect anaerobiosis (Alonso et al., 2013). In the last decades, the AFB1 content of the corn silages has been causing an increasing problem, since the area of the producing mould, the thermotolerant *A. flavus*, has started spreading in temperate zones as well. In Hungary, this mould could be cultured from 63.5% of the corn yield, and more than 40% of the isolated strains proved to be able to produce that carcinogenic mycotoxin according to the results of comprehensive examinations in the years 2009–2011 (Dobolyi et al., 2013). In order to eliminate the aflatoxin content of silages, the treatment of the initial mixture with LAB-containing preparates is used throughout the world (Fabiszewska et al., 2019). Recently, beside their lactic acid production, the cells of LAB have been recognised to be able to reduce the aflatoxin level in the silage by adsorption to their cell walls (Bata-Vidács et al., 2018).

The objectives of this study were to investigate the effect of LAB-inoculation on the level of yeast population and the taxonomic diversity of yeast communities in the experimental corn silages that were contaminated with an aflatoxin-producing *A. flavus* strain. The applied LAB preparate contained different *Lactobacillus* and *Pediococcus* strains.

2. MATERIALS AND METHODS

2.1. Experimental silos and silage samples

Ensiling was carried out in stainless steel homemade microsilo containers of 200 L of inner capacity. The initial matrix was made from whole crop corn in the middle of September, and it was chopped to pieces of 5–10 cm and was inoculated with the toxinogenic mould *A. flavus* Zt41 (NCAIM F.01021). The inoculation was carried out with 1–2% of rice grain inoculum

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made from the strain of our own isolate *A. flavus* Zt41 as follows: a mould suspension was prepared from 7-day-old colonies grown on PDA (Potato Dextrose Agar, VWR) at room temperature with sterile distilled water using a Potter homogeniser. The mould propagulum count of the suspension was 10^8 conidia mL^{-1}. Commercially available rice was sterilised by autoclaving at 121 °C. The sterile rice was inoculated with the mould suspension, then it was incubated at 26 °C for 3 weeks.

LAB strains with the highest AFB1 binding capacities from out culture collection were selected based on previous studies (Bata-Vidács et al., 2020; Kosztik et al., 2020). Inoculation with LAB culture was carried out as follows: strains *Lactobacillus salivarius* SK46, *L. plantarum* AT26, and *Pediococcus pentosaceus* OR68 stored at −20 °C were inoculated into MRS broth and incubated at 37 °C for 48 h. 4.5 mL of the suspension was inoculated into 500 mL of MRS broth, followed by another incubation at 37 °C for 48 h. The suspensions were counted with a Bürker-chamber under a microscope. The germ counts were as follows: *L. salivarius* – 6.0×10^8 cells mL^{-1}, *L. plantarum* – 1.2×10^9 cells mL^{-1} and *P. pentosaceus* – 8.0×10^8 cells mL^{-1}. All used *Lactobacillus* strains were our own isolates. The fermentation process in the silages was carried out at the temperature of 20 °C for two months. Samples from the silos were taken at days 0, 3, 7, 14, 28, and 56 for toxin measurements and yeast number determinations. Immediately after the opening of the silo, the surface layers were removed and the inner parts were sampled. Samples of 50–60 g were taken, placed into sterile plastic bags, and transported to the laboratory. Water extract suspensions of the samples were prepared immediately upon arrival by blending 10 g of silage with 90 mL of peptone water (peptone 1 g, NaCl 9 g in 1,000 mL sterile water). The samples were homogenised for 45 s in a Pulsifier instrument (Microgen Bioproducts Ltd.).

2.2. Determination of AFB1 concentration, using HPLC method

Mycotoxin was extracted from the silage models as follows: 20 g of silage was taken from each type of microsilos into Stomacher bags for toxin determination at every sampling point, 80 mL of methanol was added, then the bags were intensively shaken for 45 s with a Pulsifier device (Microgen Bioproducts Ltd.). The bags were left to stand in a dark place for 24 h, then pulsified again (45 s) for more thorough toxin extraction. The filtrates were poured into 50 mL plastic centrifuge tubes and centrifuged (Centrifuge 5810 R, Eppendorf, Vienna, Austria) at 20 °C and 3,000 r.p.m. for 10 min. 1–1 mL of the extracts were evaporated to dryness in a nitrogen atmosphere, then the derivatisation of the evaporated samples was carried out as follows: the samples were re-dissolved in 0.4 mL of n-hexane, vortexed (Vortex Genie 2, MO BIO Laboratories, Carlsbad, CA, USA) for 30 s, then 0.1 mL of TFA was added and vortexed again for 30 s. The samples were incubated at 60 °C for 15 min, cooled to room temperature, 0.4 mL H_2O:MeCN (9:1) mixture was added, and the samples were vortexed for 30 s. After the separation of the phases, the lower phase was collected, centrifuged (13,000 r.p.m., 10 min), transferred into HPLC vials, then injected into the HPLC device.

AFB1 was determined with a Shimadzu modular type HPLC system with a CBM-20A control unit connected to two LC-20AD pumps, a DGU-14A degassser, a CTO-10ASVP column thermostat, a SIL-20A automatic sample feeder, and a RF-20A fluorescent detector. Isocratic HPLC method was used for the detection and qualification of AFB1. 65:35 mixture of HPLC-grade water and metanol:acetonitrile (1:1) was used as eluent. The separation was carried

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out on a Prodigy C18 150 × 4.6 mm 5 µm column (Phenomenex, USA), flow rate was set to 1 mL min^{-1}, the volume of the injected sample was 3 µL. Excitation and emission wavelengths were adjusted to 350 and 430 nm, respectively, while the column thermostat temperature was set to 40 °C. A factory-purified aflatoxin (B1, B2, G1, and G2) mixture (Sigma Aldrich Corporation, St. Louis, Missouri, US) was used as a standard during the determination. Collection and evaluation were performed with the ClassVP v6.14 program.

2.3. Testing genotoxicity

The SOS-Chromotest kit was purchased from Environmental Bio-Detection Products Inc (Mississauga, Ontario, Canada), and used based on the recommendations (Risa et al., 2018) and optimised guidelines of the manufacturer. The test was implemented in white, sterile, 96-well micro-titre plates (µClear®, Greiner Bio-One Hungary Ltd., Mosonmagyaróvár, Hungary). Enzyme activity was determined photometrically with Filter Max 400 microplate reader.

As blank, 100 µL sterile casitone broth was used. As positive controls, 4-nitroquinoline 1-oxide (4-NQO, direct genotoxin) and 2-aminoanthracene (2AA indirect genotoxin) were used in two-fold serial dilutions. As negative control, 10% DMSO in saline was applied. In the study, 10 µL toxin solutions extracted from the different types of model silages at the end of the experiment (on the 60th day) were measured in 3 parallels. 100 µL suspensions of the test organisms was added to the samples and to the positive and negative controls both.

As test organism, *E. coli* PQ37 was cultured overnight in casitone broth (2.5 g yeast extract, 2.5 g casitone peptone, 8.5 g NaCl in 1L distilled water), the optical densities of the suspensions were adjusted to OD_{600} = 0.05. The test plate was incubated in the dark at 37 °C for 120 min. After the addition of 100 µL substrate mix (Blue Chromogen 10 mL and 50 mg *p*-nitrophenyl –phosphate dilution) to each well, a second incubation was carried out in the dark at 37 °C for 120 min. The strength of the developed colour depended on the genotoxicity of the samples. The colours were measured with the Filter Max 400 microplate reader at 405 and 620 nm wavelengths.

Based on the determined values, Induction Factor (IF) was calculated from the absorbance values, which is indicative of the genotoxicity:

$$\mathrm{IF} = \frac{A_{405}\ nc \times A_{620}\ t)}{(A_{405} t \times A_{620} nc)}$$

nc = negative control
t = test sample
A_{405} = absorbance level at 405 nm wavelength
A_{620} = absorbance level at 620 nm wavelength

In case of the IF > 1.5, the tested sample has genotoxic effect on the test organism.

SOS-inducing potential (SOSIP) was determined as a validation of the results, which is a single parameter representing the IF per mass unit of a test compound (Quillardet and Hofnung, 1985). The value was determined from the slope of the dose-response curve, additionally using the values of the positive control (4NQO). 4NQO was determined as 68.51 along with the correction factor (CF = 0.96) by Csenki et al. (2022).

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2.4. Enumeration and isolation of yeasts

For quantitative culturing of yeasts, the obtained water extract suspensions were passed through sterile plastic tea-filters. Immediately after an intensive shaking, aliquots of 1 mL of suspensions were taken and 10-fold serially diluted with peptone water. Aliquots of 0.1 mL of each step of dilutions were plated on rose bengal chloramphenicol agar (Scharlau, Barcelona, Cataluña). After incubation at 25 °C for 4–5 days, yeast colonies were enumerated, all yeast colony counts were transformed to $\log_{10}$ CFU g^{-1}, and expressed on a fresh weight basis. Between 15 and 20% of the yeast colonies were randomly picked from the plates containing >20 colonies. The picks were streaked onto PDA, incubated at 25 °C for 5 days, and a single colony as an isolate was recovered. The colony purification process was repeated 2 more times, then the isolates were suspended in 20% glycerol solution and frozen at −80 °C for maintenance. The isolates were typified according to their phenotypic features, and the representatives of each phenotype were identified with molecular methods.

2.5. DNA extraction and gene sequencing

The yeast species selected based on their morphological features were grown on PDA plates for 4 days. After the incubation period, DNA extraction was implemented on the colonies by using Masterpure ™ Yeast DNA Purification Kit (Epicentre Bio-technologies, Madison, WI, US). The DNA fragments of the species were amplified with the following primers: ITS1, ITS4, NL1, and NL4. The sequences of the primers are found in Table 1.

Amplicon sequencing was performed by BaseClear Inc. (Leiden, The Netherlands). The electropherograms were evaluated with MEGA6 program, sequences were identified with blast analysis via BLAST engine of GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A phylogeny tree was built by using the base-sequences, which shows the family relationships and distance between each species.

2.6. Statistical analysis

Microbial counts were $\log_{10}$–transformed to obtain normally distributed data and were presented on a wet basis. The significant differences between the means were identified from the *P*-values and the effects were considered significant at $P < 0.05$. Due to the heterogenic variance of data, specific variances were used for analysis. Data on yeast identification and distribution were explored using descriptive analysis. Redundancy and taxonomy data were imported into phyloseq object in R (version 4.2.2) and plots were generated with phyloseq package (version 1.40.0).

Table 1. For internal transcribed space (ITS) rDNA region amplification forward ITS-1 and reverse ITS-4 primers were used. The D1/D2 domain of 26S rDNA region was amplified using the primers NL1 and NL4

Gene name	Primer sequences
ITS1	**5′**-TCCGTAGGTGAACCTGCGG-**3′**
ITS4	**5′**-TCCTCCGCTTATTGATATGC-**3′**
NL1	**5′**-GCATATCAATAAGCGGAGGAAAAG-**3′**
NL4	**5′**-CCGTCTTGAAACACGGACC-**3′**

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3. RESULTS AND DISCUSSION

3.1. Aflatoxin B1 content of the experimental crop corn silages

Inoculation with the mycotoxigenic strain *A. flavus* Zt41 resulted a definitely high AFB1 content in the experimental CSCA, while the untreated control CS remained toxin-free along the experimental period (Fig. 1). The concentration of AFB1 produced as a secondary metabolite started to increase significantly from day 14, even though the natural lactobacillus and propionibacillus communities of the silage exerted their well-known inhibitory effects. The AFB1 content of CSCA inoculated with LAB was 5–8% lower in the first week, the difference increased steadily from day 14 onwards, and after 8 weeks the difference was more than 40%. The cause of the decrease in aflatoxin levels, whether it was caused by direct toxin degradation or by inhibition of toxin production, may be further investigated by using toxin containing, aspergillus-free control.

3.2. Genotoxicity of the experimental crop corn silages

The crop corn silage experimentally inoculated with the toxinogenic *A. flavus* Zt41 strain (CSCA) showed a high induction factor (IF: 2.2) with SOS Chromotest after an 8-week incubation period (Fig. 2). After the same incubation period, only a notably lower induction factor

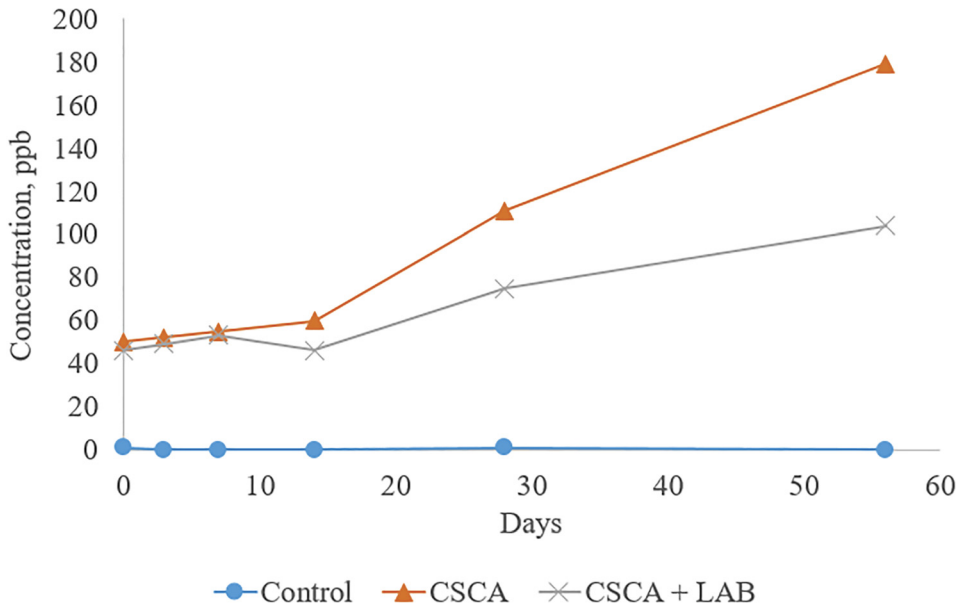

Fig. 1. Effect of LAB inoculation on the AFB1 content of the crop corn silage inoculated with the toxinogenic strain *Aspergillus flavus* Zt41 (CSCA: crop corn silage contaminated with *A. flavus* Zt41; CSCA+LAB: crop corn silage contaminated with *A. flavus* Zt41 inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68)

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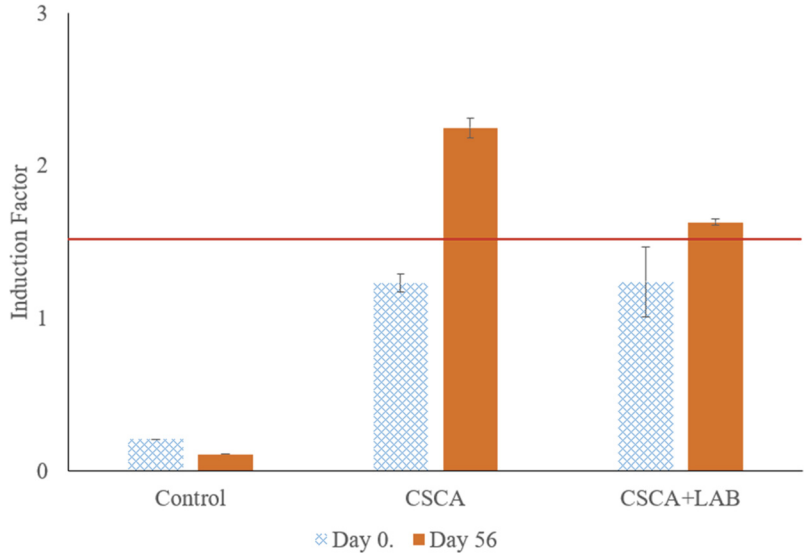

Fig. 2. Induction factors representing genotoxicity of the experimental crop corn silages (CSCA: crop corn silage contaminated with *Aspergillus flavus* Zt41; CSCA+LAB: crop corn silage contaminated with *A. flavus* Zt41 inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68; red line represent IF = 1.5; $n = 3$)

(IF: 1.6) was observed for CSCA+LAB, indicating a rather lower level of genotoxicity. It should be noted that the induction factor of the starter cropped corn was low (IF: 0.21), which means that it was not genotoxic. After 8 weeks, the induction factor of untreated silage was even lower (0.11), also not in the genotoxic range. SOS-Chromotest is not an aflatoxin-specific test, any mutation-causing material in the silage can trigger it. It was not possible to analyse the composition of the silo in this project and finding other genotoxic substances than AFB1. IF value of 1.1 is not genotoxic and not very different from the levels that can be found in e.g. pesticide-loaded soil samples.

3.3. Total quantities of culturable yeasts

Remarkable yeast populations were present in the whole crop corn silage and varied significantly during the fermentation process (Fig. 3). The initial mass of the chopped crop corn mixture contained significant amounts of yeast propagula (3.3×10^7 CFU g^{-1}). The untreated control silage showed an order of magnitude decrease in yeast count from days 7–14. Between days 14 and 28, the quantity of yeast propagula decreased exponentially, declining the level 2.8×10^2 CFU g^{-1} by day 56. During the fermentation process, the quantity of yeast propagula of CSCA was slightly lower than that of the untreated CS, and declined to 95 CFU g^{-1} on day 56. The yeast content of CSCA+LAB was lower than that of the untreated control silage at all times during fermentation: by about 50% at day 14 and by an order of magnitude at days 28 and day 56.

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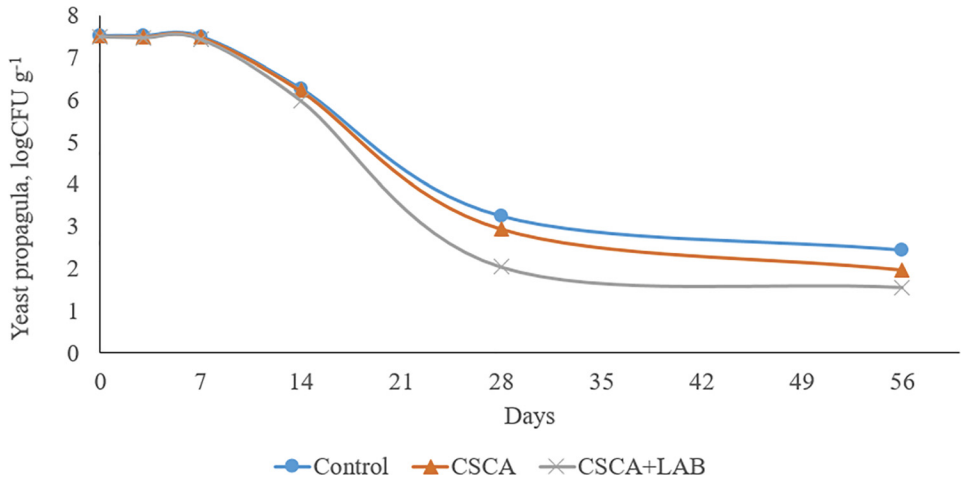

Fig. 3. Dynamic changes of yeast populations of the untreated whole crop corn silage (Control), silage contaminated with toxinogenic *Aspergillus flavus* Zt41 (CSCA), and silage contaminated with toxinogenic *A. flavus* Zt41 and inoculated with LAB (CSCA+LAB) during the fermentation process

3.4. Distribution of yeast species

144 strains were randomly isolated from the initial whole crop corn mixture, 132 from the uninoculated control CSCA, and 158 from the CSCA+LAB. The strains finally selected by microscopy and colony morphology to represent the yeast communities were obtained from different samples (Table 2).

Based on the DNA sequences, the selected strains belonged to different groups. Maximum-parsimony analysis of the concatenated ITS and D1/D2 sequences placed three representative strains, K14, K11, and Sd210, into the basidiomycetous species *Cryptococcus carnescens*,

Table 2. Origin of the strains isolated from the experimental silage samples

Laboratory code	Origin	Species
K11	CS	*Cystobasidium slooffiae*
K14	CS	*Cryptococcus carnescens*
Sd059	CS	*Wickerhamomyces anomalus*
Sd157	CSCA+LAB	*Meyerozyma guilliermondii*
Sd158	CSCA	*Pichia kudriavzevii*
Sd210	CS	*Clavispora lusitaniae*
Sd215	CSCA	*Saccharomyces cerevisiae*
Sd217	CS	*Pichia fermentans*
Sd226	CS	*Kregervanrija fluxuum*

CS: control crop corn silage; CSCA: crop corn silage contaminated with *Aspergillus flavus* Zt41; CSCA+LAB: crop corn silage contaminated with *A. flavus* Zt41 inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68.

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Cystobasidium slooffiae, and *Clavispora lusitaniae*, respectively. The other 6 strains, Sd059, Sd157, Sd215, Sd226, Sd217, and Sd158, belonged to the ascomycetous species *Wickerhamomyces anomalus, Meyerozyma guilliermondii, Saccharomyces cerevisiae, Kregervanrija fluxuum, Pichia fermentans*, and *Pichia kudriavzevii*, respectively (Fig. 4).

Relative abundance of the yeast species greatly differed in the experimental corn silages. Four of the total 8 species (*C. lusitaniae, Cr. carnescens, Cys. slooffiae,* and *W. anomalus*), were found only in the initial crop corn silage, but 2 of them (*M. guilliermondii* and *S. cerevisiae*) occurred in all samples. While the number of species decreased from 8 to 4 during the control ensiling, the incidence of 3 species (*S. cerevisiae, P. kudriavzevii,* and *M. guilliermondii*) noticeably increased. Similarly, only 4 species could be cultured from the CSCA+LAB, and 2 of them (*S. cerevisiae* and *M. guilliermondii*) at higher incidence (51.3% and 30.4%) (Fig. 5).

3.5. Diversity of yeast communities

The zymomycota of the initial crop corn silage consisted of 8 species: *Cr. carnescens* (26.4%), *Cys. slooffiae* (19.4%), *C. lusitaniae* (18.1%), *K. fluxuum* (17.4%), and *M. guilliermondii, P. fermentans, S. cerevisiae,* and *W. anomalus* with lower incidences. However, the zymomycota CSCA consisted of only 4 species: *S. cerevisiae (37.9%), P. kudriavzevii* (37.1%), *M. guilliermondii* (21.2%), and *P. fermentans* (3.4%). Interestingly, the zymomycota of CSCA+LAB comprised also 4 species: *S. cerevisiae* (51.3%), *M. guilliermondii* (30.4%), *K. fluxuum* (13.3%), and *P. kudriavzevii* (5.1%) (Table 3).

The yeast community of the initial crop corn silage, due to the 8 culturable species, showed considerably high alpha diversity values of 1.827, 0.812, and 1.834 as Shannon's, Simpson's, and Fisher's indices, respectively. The yeast communities of the silages considerably differed from that of the initial silage. CSCA showed significantly lower alpha diversity values of 1.188, 0.715, and 0.778 as Shannon's, Simpson's, and Fisher's indices, respectively. Diversity of the CSCA+LAB's yeast community was found to be even lower with alpha diversity values of 1.123, 0.573, and 0.525 as Shannon's, Simpson's, and Fisher's indices, respectively (Fig. 6).

The 50–55 ppb AFB1 content of CSCA measured in the first 7 days suggests that the residual mycotoxin adsorbed in the cell wall of the mould conidia is already being released into the substrate as a result of the initial metabolism, i.e., this initial aflatoxin is not yet a secondary product. The latter effects can be explained by the faster growth of the used, i.e., the early appearance of a surface capable of binding the toxin. From day 14 onwards, the lower AFB1 content of silage inoculated with LAB is presumably the result of a more complex mechanism, in which, in addition to the toxin-binding and amensalistic effects of LAB, their increased lactic acid production and the higher lactic acid content of the medium must be a significant factor inhibiting the growth of aspergilli, all in a perfectly anaerobic environment.

The genotoxic effect on the living environment and, in the case of silage, on humans via milk was clearly detectable by SOS Chromotest in *A. flavus*-contaminated silage, as indicated by the high induction factor (IF: 2.2). The effect of the inoculation with LAB was also observed by a reduction in the genotoxicity of CSCA+LAB (IF: 1.6), but it should be noted that the latter value indicates even milder genotoxicity using Walker's (1987) calculation.

The primary reason for the high yeast content of the initial crop silage was the inherently high yeast community in the corn phyllosphere, as it has been reported by Lindow and Brandl (2003) and Nazar et al. (2022). This may have been further contributed to by the fact that the harvest

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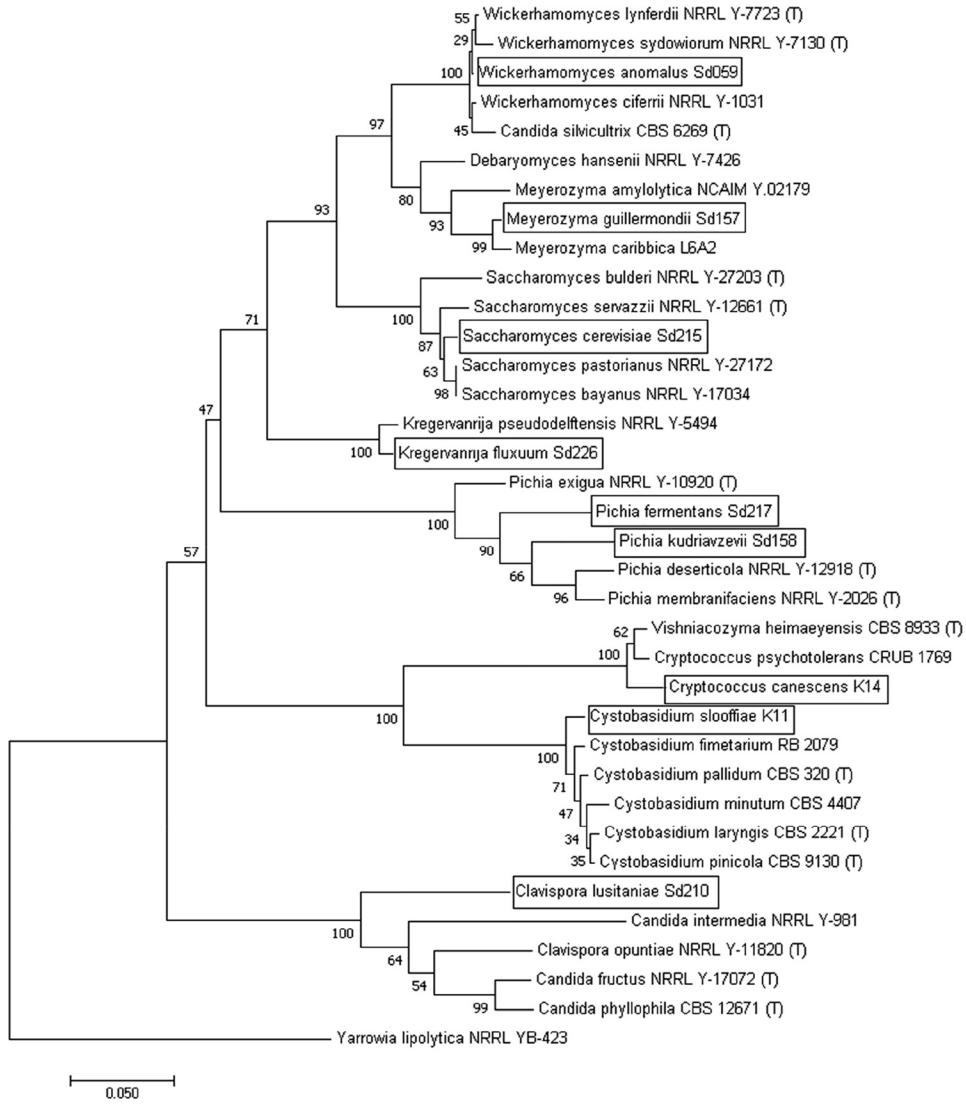

Fig. 4. One of three most parsimonious trees showing the placement of the yeast species isolated from the crop corn silage, crop corn silage inoculated with *Aspergillus flavus* Zt41, and crop corn silage inoculated with *A. flavus* Zt41 and with LAB strains strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68. The analysis was based on the concatenated sequences of internal transcribed spacer (ITS) regions and D1/D2 domains of the large subunit rRNA gene. The Maximum Parsimony tree was obtained with Subtree-Pruning Regrafting (STR) algorithm (Nei and Kumar, 2000). *Y. lipolytica* was used as a designated outgroup. The bar indicates 50 bases substitution

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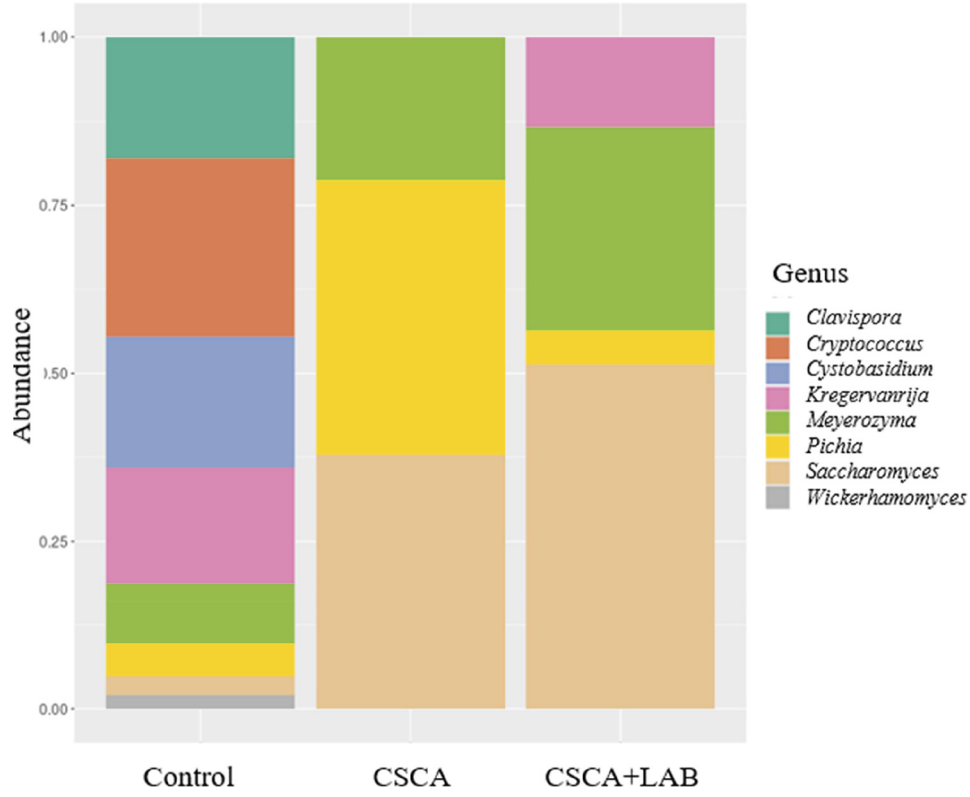

Fig. 5. Relative abundance of yeast species at genus level (CSCA: crop corn silage contaminated with *Aspergillus flavus* Zt41; CSCA+LAB: crop corn silage contaminated with *A. flavus* Zt41 inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68)

and chop of the whole crop corn were carried out in dry weather, so that neither the tissue fluids that were released on the cutting or crushing surfaces, nor the starting yeast community could be diluted by precipitation water. It should be noted that at the start of the experiment, in mid-September, the temperature of the mixture (23–25 °C) was favourable for the yeasts.

During the ripening process in natural, untreated whole crop corn silage, a dynamic interaction of microbial communities takes place. The most typical groups of the silage microbiota are LAB and those yeast species that are tolerant to acidic and anaerobic environments and capable of lactate assimilation. The levels of yeast communities in control crop corn silage experimentally produced in microsilos in our experiments were in agreement with the results published worldwide (Borreani et al., 2018; Zhang et al., 2020; Parra et al., 2021). It is well known that the fast-growing LAB use up easily available nutrients quickly, and that yeasts can grow more slowly in nutrient-poor environments. The lactic acid and propionic acid produced by the lactic acid and propionic acid bacteria make the silage highly acidic, and the concomitant production of CO_2 makes the medium anaerobic, which also inhibits the growth of yeasts (da Silva et al., 2024).

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Table 3. Frequency of yeast species isolated from the crop corn silage (CS), crop corn silage contaminated with *Aspergillus flavus* Zt41 (CSCA), and crop corn silage contaminated with *A. flavus* Zt41 and inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68 (CSCA+LAB)

Species	CS		Silage after 8 weeks			
			CSCA		CSCA+LAB	
	cc	%	cc	%	cc	%
Clavispora lusitaniae	26	18.1	0	0	0	0
Cryptococcus carnescens	38	26.4	0	0	0	0
Cystobasidium slooffiae	28	19.4	0	0	0	0
Kregervanrija fluxuum	25	17.4	0	0	21	13.3
Meyerozyma guilliermondii	13	9.0	28	21.2	48	30.4
Pichia fermentans	7	4.9	5	3.4	0	0
Pichia kudriavzevii	0	0	49	37.1	8	5.1
Saccharomyces cerevisiae	4	2.8	50	37.9	81	51.3
Wickerhamomyces anomalus	3	2.1	0	0	0	0

cc: colony count

Quantity of yeast propagula of the CSCA during fermentation was slightly lower than that of the untreated CS, and was 95 CFU g^{-1} on day 56. The yeast content of CSCA+LAB was the lowest of the three settings during fermentation, being about 50% lower than the untreated control on day 14 and an order of magnitude lower on days 28 and 56.

The yeast isolates obtained from the CS represented 8 different species. The most frequent 2 of them belonged to the non-fermentative basidiomycetous species *Cr. carnescens* and *Cys. slooffiae*, similar to findings of Sohrabi et al. (2023). The ability of the latter to utilise a wide range of soluble carbon sources makes them likely to be typical inhabitants of the phyllosphere. Two other yeast species (*C. lusitaniae* and *M. guilliermondii*) were isolated also in larger proportions. It is interesting to note that in the yeast community of the initial cropped corn with a high diversity, we also found species with good fermentative ability (*P. fermentans, S. cerevisiae,* and *W. anomalus*), although in a smaller proportion, but similar to some other authors' observations (Into et al., 2020). *S. cerevisiae* is competitive only in sugar-containing liquid habitats (Watanabe and Hashimoto, 2023), so its presence in the initial cropped corn, even at such low levels, was unexpected. The 4 yeast species of the control CS all have good fermenting abilities and are able to assimilate lactate, which is formed in high amounts in silage. It is assumed that *S. cerevisiae* and *Pichia* species produce ascospores in addition to their vegetative growth due to the limited sugar concentration and the high acetate content of the medium (Bai et al., 2021). It can also be seen that the dominant obligate aerobic species in the initial cropped corn have perished or their numbers have fallen below the limit of detection. The LAB treatment has caused a significant change in the species composition of the yeast community, but the small number of species makes it difficult to evaluate the change, as the appearance or disappearance of a species can be influenced by a number of environmental factors. According to the authors (Santos et al., 2017; Wali et al., 2022) investigating yeast diversity in silages, the abundance of the most common genus, *Saccharomyces*, was notably increased by LAB treatment, while the proportion of strains belonging to the genus *Pichia* decreased. In our consideration, the presence of two

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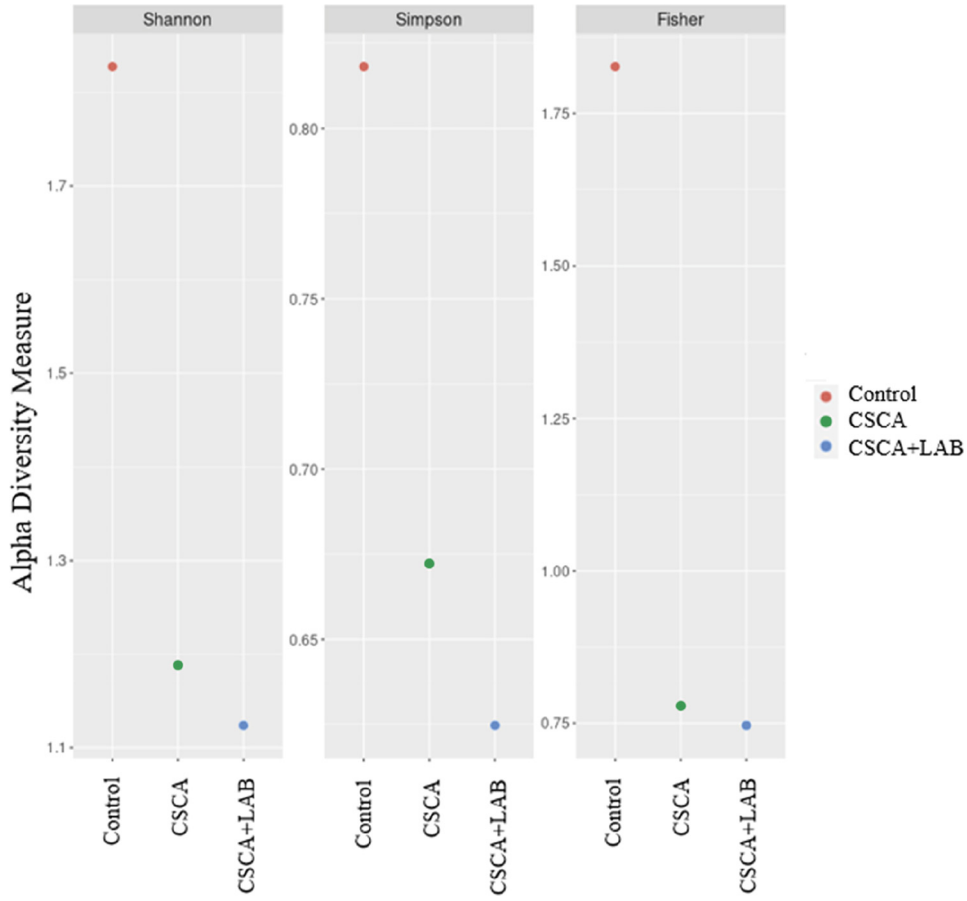

Fig. 6. Alpha diversity values of the yeast communities of the experimental corn silages by Shannon's, Simpson's and Fisher's indices (CSCA: crop corn silage contaminated with *Aspergillus flavus* Zt41; CSCA+LAB: crop corn silage contaminated with *A. flavus* Zt41 inoculated with LAB strains *L. salivarius* SK46, *L. plantarum* AT26 and *P. pentosaceus* OR68)

additional species, *K. fluxuum* and *M. guilliermondii*, and the changes in their proportions could be influenced by the active presence of the toxin-producing mould *A. flavus*, as also reported by Kalúzová et al. (2022).

4. CONCLUSIONS

Considering the increasing contamination of silages with aflatoxins worldwide, the effect of LAB inoculation was investigated in corn silage experimentally contaminated with a toxigenic *A. flavus* strain. From our results, it can be concluded that the effect of a LAB strain mixture applied as a starter culture in crop corn silage containing the physiologically active mould

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A. flavus does not alter the typical yeast communities of silages in terms of either the population size or the presence of dominant species.

In addition to the analytical determination of the toxin content of the silos contaminated with aflatoxin-producing moulds, we also performed SOS Chromotest-based analysis of the samples. Microbial inoculant used for detoxification may be able to metabolise the toxin, which may result in a reduction of the toxin content and the production of additional, even more toxic metabolites. Krifaton et al. (2011) has shown such an effect with AFB1-degrading soil microbes and underlined the necessity to employ bio-tests in biodetoxification assays, as cytotoxicity and/or genotoxicity may occur even after substantial degradation. In our study, SOS Chromotest results as IF = 0.11 for control, IF = 2.2 for *A. flavus* contraminated and IF = 1.6 for *A. flavus* contraminated and LAB treated silos were received. Considering the procedure of Walker (1987), who stated that above 1.5 IF the sample was genotoxic, the silage in our study contaminated with toxinogenic *A. flavus* can be referred to as higly genotoxic, and a low genotoxicity was measured in the LAB-treated sample. According to these results, LAB can reduce AFB1 by their detoxifying or binding capabilities, but further studies are required to assess such capacity in practice and at different levels of AFB1 contamination.

Conflict of interest: I. Bata-Vidács is a member of the Editorial Board of the journal, therefore, she did not take part in the review process in any capacity and the submission was handled by a different member of the editorial board. The submission was subject to the same process as any other manuscript and editorial board membership had no influence on editorial consideration and the final decision.

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