Doctoral (PhD) thesis

MYCOTOXIN ELIMINATION BY MICROORGANISMS OF SILAGE AND FOOD INDUSTRY ORIGIN

Cintia Adácsi

Supervisor: Tünde Pusztahelyi PhD

UNIVERSITY OF DEBRECEN

Doctoral School of Nutrition and Food Sciences

Debrecen, 2024

1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL THESIS

The mycotoxins produced by certain fungi are dangerous for humans and animals and pose a biological hazard due to their presence in food and feed. Mycotoxins are found at every step of the food chain and can cross different levels of the food pyramid. The entry of mycotoxins into the food chain is simple: mycotoxins enter the human body both directly and indirectly through cereals (Figure 1). Their adverse effects can be directly detected in humans through consuming agricultural crop-based products, animal feed contaminated with mycotoxins, and animal products. Food of animal origin contaminated with mycotoxins and milk and dairy products containing aflatoxin M1 (AFM1) and ochratoxin A (OTA), accumulated in meat pose a risk to human health.

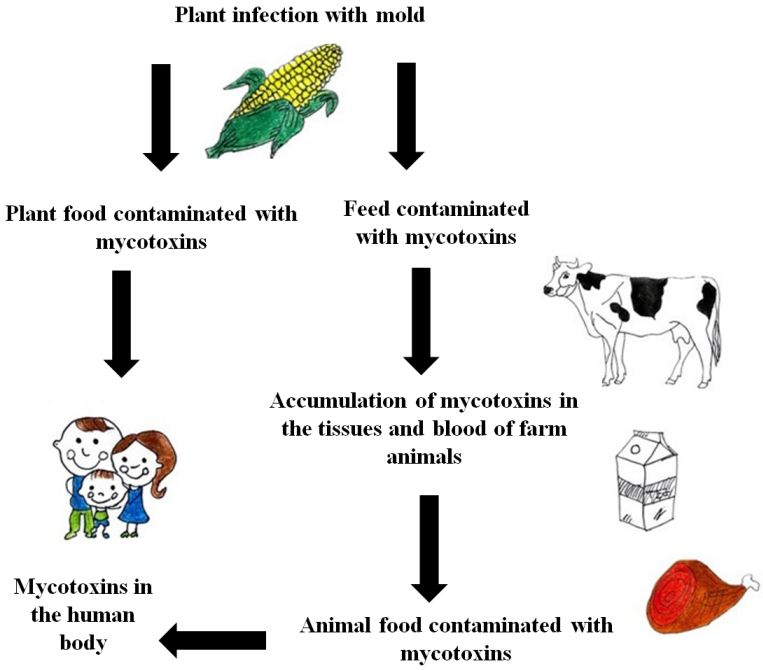


Figure 1 Mycotoxins in the food chain (self-edited)

My doctoral thesis aims to highlight the value of biological detoxification and the usefulness of bacteria in these processes.

In my thesis, I set myself the following goal:

-determination of the microbiological status of fermented feedingstuffs (total microbial count, lactic acid bacteria (LAB), moulds and mesophilic sulphite-reducing *Clostridium* count),

-detection of mycotoxin contamination of fermented feedingstuffs by HPLC (High-Performance Liquid Chromatography),

-isolation and characterisation of bacteria with high mycotoxin resistance and elimination,
-identification of isolated bacteria based on 16S rRNA and MALDI-TOF MS (matrix-assisted laser desorption ionisation coupled to time-of-flight mass spectrometry),
-testing of mycotoxin elimination potential of silage feed and dairy organisms (live and bacterial cell fractions) by HPLC and ELISA (enzyme-linked immunosorbent assay) from the perspective of possible future use,
-making silage from maize plants collected by me for mycotoxin elimination testing.

2. MATERIALS AND METHODS

2.1. Materials

The fermented feed samples examined were maize silage, rye silage, rye haylage, alfalfa silage, alfalfa haylage, and triticale haylage (in 2019-2020 in Hungary, from dairy cattle farms in Hajdú-Bihar county) (Adácsi et al., 2022a).

The raw milk samples came from randomly different husbandries. 100 ml of raw milk was collected from the tankers at the collection point of Alföldi Tej Kft. (Debrecen). The milk samples were delivered chilled.

Lactiplantibacillus plantarum NCAIM strain B01074 has been provided by the National Collection of Agricultural and Industrial Micro-organisms (MATE Institute of Food Science and Technology).

Lactococcus lactis ssp. lactis R703, *Bifidobacterium animalis ssp. lactis* BB12, L. *Paracasei ssp. paracasei* 431 (Chr. Hansen A/S, Denmark) strains were grown in MRS broth (Scharlau, Germany) for 24 hours at 30 °C. The bacterial cultures were centrifuged at 6081 g (centripetal acceleration) for 10 minutes (4 °C) under sterile conditions to remove the supernatant. The collected biomass was washed with PBS (phosphate-buffered saline) (Sigma-Aldrich) and divided into 100 µl aliquot parts in Eppendorf tubes for treatments. The cell concentration was 10^9 TKE/ml. I stored the aliquot portions at -18 °C.

The maize crop used for in vitro silage production was Sy Orpheus maize grown in the DE MEK Demonstration Garden, which was assisted in its cultivation by the DE MEK Precision Crop Production Research and Development Service Center.

2.2. Microbiological analysis

100 g of silage sample was suspended in sterile homogenising Stomacher bags in buffered peptone water (BPW) (Scharlab, Barcelona, Spain) and homogenised with a Stomacher homogeniser (IUL Instruments, Barcelona, Spain). Then, further decimal dilutions were made from the suspensions.

The total microbial count was determined using a plate count of Agar (PCA) (Scharlab, Barcelona, Spain) medium through a deep inoculation process. Incubation occurred at 30 °C for three days under aerobic or anaerobic conditions (ISO 4833-1:2013). To provide anaerobic space, an Oxoid™ AnaeroGen™ 2.5L sachet (Thermo Scientific™) was used in an anaerobic vessel (Anaerocult, Merck).

The number of lactic acid bacteria was determined by surface spreading on De Mann-Rogosa-Sharp (MRS) agar (Scharlab, Barcelona, Spain) plates. The plates were incubated at 30 °C under anaerobic conditions for three days.

Moulds were determined by surface dispersion on chloramphenicol-glucose-agar (CGA) (Scharlab, Barcelona, Spain) medium, which was incubated at 25 °C for five days.

The sulfate-reducing *Clostridium* count was determined using the Iron Sulfite-Agar (Scharlab, Barcelona, Spain) medium through a deep inoculation process. The solid medium was anaerobically incubated at 37 °C for three days.

2.3. Mycotoxin detection

2.3.1. Mycotoxin detection by HPLC methods

Biopure mycotoxin standard solutions (Romer Labs, Tulln, Austria) were used in appropriate dilution for HPLC measurements. The samples were prepared using the VICAM (Waters) method with some modifications.

HPLC was performed on Hitachi Elite LaChrom HPLC (San Jose, CA, USA) to detect deoxynivalenol (DON). Phenomenex (Torrance, CA, USA) RP-C18 column (125x4 mm, 5 μm) was used and detected with a diode array (DAD) detector at UV 218 nm with acetonitrile: water (10:90) eluent. In the elimination test, filtered supernatant samples were measured in HPLC.

To detect DON from silage, 12.5 g of dried sample was homogenized with 2.5 g polyethylene glycol (VWR International Ltd., Hungary) and 50 ml distilled water under high-speed stirring. After filtering, 1 ml of extract was poured onto the DON

immunoaffinity column (VICAM DONtest HPLC column, Weber Consulting Ltd.). The column was washed successively with 5 ml of distilled water and 5 ml of methanol to elute the toxin from the column. The methanol was evaporated from the samples on Rotagest (Büchi).

The detection of aflatoxins (AF) and ochratoxin A (OTA) HPLC was performed with Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA). For AF detection, a Phenomenex (Torrance, CA, USA) RP-C18 column (150 x 4.6 mm, 5 μm) was used, with a Romer UV derivative-forming unit (Romer Labs Ltd., Tulln, Austria) and fluorescence detector (ex360 nm, em440 nm), methanol: water (45:55) eluent. For OTA detection, Phenomenex RP-C18 column (150*4.6 mm, 5 μm) was used with a fluorescence detector (ex360 nm, em440 nm), acetonitrile: 0.012 M Na-phosphate pH 7.5 (60:40) eluent.

In the aflatoxin B1 (AFB1) mycotoxin elimination test, a high-speed vortexed sample mixed with methanol in a 1:1 ratio was measured.

To detect AF and OTA in silage, 25 g of dried sample was homogenised with 2.5 g sodium chloride (VWR) and 50 ml 80% methanol (HPLC, Sigma-Aldrich) under high-speed stirring. The extract was diluted with distilled water in a ratio of 1:4. The diluted extract was filtered, and 10 ml was filled onto the immunoaffinity column (VICAM AflaTest HPLC columns, Weber Consulting Ltd.) or the ochratoxin immunoaffinity column (VICAM OchraTest WB HPLC column, Weber Consulting Ltd.). The column was washed with 10 ml of distilled water, and the toxin was eluted with 5 ml of methanol.

For the detection of zearalenone (ZEA), HPLC was performed according to Dionex Ultimate 3000 (Thermo Scientific). A Phenomenex (Torrance, CA, USA) RP-C18 column (150 x 4.6 mm, 5 μm) was used with a fluorescence detector (ex274 nm, em440 nm) and acetonitrile-water: methanol (46: 46:8) eluent.

In samples exposed to ZEA, the mycotoxin content of supernatants and pellets was also measured. The supernatants were treated with methanol in a 1:1 ratio and vortex at high speed. The pellet adsorbed ZEA was extracted with acetonitrile-water-methanol (46:46:8).

To measure ZEA from solid samples, 20 g of dried sample was homogenised with 2 g sodium chloride (VWR International, Hungary) and 50 ml 90% acetonitrile (HPLC, Sigma-Aldrich) while stirring at high speed. The extract was diluted with distilled water in a ratio of 1:4. The diluted extract was filtered, and 10 ml was filled onto the zearalenone immunoaffinity column (VICAM ZearalaTest WB HPLC column, Weber Consulting Ltd.). The column was washed with 10 ml of distilled water, and the toxin was eluted with 5 ml of methanol.

The performance, LOD value, linear range and reproducibility of the applied HPLC methods were determined by contamination of dried and ground maize silage with mycotoxins of different concentrations (n = 8). Linear ranges up to 300 μg/kg were found for AFB1 and OTA, while linearities up to 50 mg/kg were found for DON and ZEA. The relative standard deviation was calculated as the absolute value of the coefficient of variation, and in all cases, it was found to be below 10%.

2.3.2. Mycotoxin detection in ELISA system

AFM1 detection was performed using direct competitive ELISA (Aflatoxin M1 High Sensitivity ELISA; Romer Labs, Tulln, Austria). The reference row and defatted milk were pipetted into the grooves of the microtitre plate. This was followed by a 45-minute incubation. At the end of the incubation period, I removed the liquid from the holes and washed it with Wash Solution Buffer. Aflatoxin M1 High Sensitivity Conjugate (M1 Antibody) was then applied. After an incubation period of 15 minutes, after further washing steps, the M1 substrate was applied. Then, after another 15 minutes of incubation, I stopped washing but added Stop Solution, thus stopping the enzyme reaction. Subsequently, using a Synergy HTX Multimode Reader (BioTek), samples were measured at 450 nm (n = 4, CV% $\leq$ 5%).

2.4. Isolation of bacterial strains followed by MALDI-TOF MS and 16S rRNA-based identification

31 bacterial isolates were collected from fermented feed samples. To produce a clean culture, individual colonies were quenched and then reinoculated from plates to determine total microbial counts (PCAs) and lactic acid bacteria count (MRS agar). The isolates were stored at -76 °C in glycerol and pearls (Cryoinstant Blue 50 Cryotubes Preservation system, VWR). The isolates were examined in MRS (Scharlab) and Nutrient Broth (Scharlab, Barcelona, Spain).

The isolated bacteria were identified with the help of MALDI-TOF MS (Microflex LT, Bruker Daltonik GmbH, Germany) by Dr. Zsuzsanna Rita Zudorné Dombrádi (University of Debrecen, DE Clinical Center (DEKK), Health Service Units, Diagnostic Units, Medical Microbiology). Individual colonies were analyzed using Biotyper 3.0 software (Bruker Daltonik GmbH, Germany) and its reference library version 3.1.2.

The DNA extraction protocol used to isolate genomic DNA (Wilson, 2001) was as follows: 200 μl of 16 hour culture was mixed with 1000 μl CTAB (Biochemica) lysis buffer {2%

w/v Cetrimonium bromide CTAB, 1.4 M NaCl, 100 mM Tris/HCl, 20 mM EDTA, pH 8.0} (Applichem GmbH, Darmstadt, Germany) and incubated for 30 minutes at 65 °C. After that a sample of 2 ml Lysing Matrix B (MP Biomedicals Germany GmbH, Schwege, Germany) I put it in exploratory tubes. The pearl-based lysis was performed for 20 seconds at 4000 g (centripetal acceleration) with Precellys 24 homogeniser (Peqlab Biotechnologie Gmbh, Erlangen, Germany), followed by 10 minutes of centrifugation of 18600 g (centripetal acceleration). 240 µl chloroform was added to a 600 µl supernatant, stirred for 30 seconds, and centrifuged for 20 minutes using 18600 g (centripetal acceleration). The upper aqueous phase of 400 µl was mixed with 400 µl isopropanol and centrifuged for 10 minutes using 18600 g (centripetal acceleration). After discharging the supernatant, 500 µl of 70% ethanol was added and centrifuged for 10 minutes using 18600 g (centripetal acceleration). After pouring the supernatant, 2 minutes of centrifugation of 18600 g (centripetal acceleration) followed, and the liquid collected at the bottom of the tube was removed with a small pipette and the pellet allowed to dry at room temperature. The dry DNA pellet was suspended in 500 µl sterile water.

During the DNA cleaning protocol, the DNA extract was purified on an Amicon Ultra membrane (Merck Millipore, Darmstadt, Germany). 500 µl genomic DNA extract was pipette into the Amicon Ultra screening device and centrifuged for 10 minutes using 18600 g (centripetal acceleration). As a washing step, 480 µl of water was added and 10 minutes of centrifugation of 18600 g (centripetal acceleration) followed. Inverting the Amicon Ultra filter device, centrifugation took place at 2 minutes1000 g (centripetal acceleration) and the concentrated sample was transferred from the device to the tube. After filling it with 20 µl of water, I obtained the purified DNA extract, which was stored at 4 °C.

The PCR reaction was performed with iProof High-Fidelity PCR Kit (BIO-RAD Ltd., Hercules, CA, USA Lithuania). I used genomic DNA as a template for the polymerase chain reaction. For the reaction, primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') were used (Pradhan and Tamang, 2019). 4 µl 5xiProof HF Buffer, 0.4 µl dNTP mix, 2 µl forward, 2 µl reverse primer, 1 µl DNA template, 10.4 µl distilled water, 0.2 µl iProof DNA Polymerase were pipetted into sterile PCR tubes to obtain a final volume of 20 µl. PCR was performed at 98 °C for 3 minutes; then at 98 °C for 30 seconds, at 54 °C for 30 seconds, at 72 °C for 45 seconds for 30 cycles and at 72 °C for 7 minutes on a heat cycle T100 (T100 Thermal Cycler, BIO-RAD Ltd., Lithuania).

DNA was cleaned using the Nucleo SpinGel and PCR Clean up Column, Macherey-Nagel, Düren, Germany, according to the protocol specified by the manufacturer. I poured 0.8% BIOLINE agarose (Meridian Life Science, Memphis, Tennessee, USA) gel, which contains: 50x TAE buffer, distilled water. I applied 10 µl of DNA sample to the pockets of the gel. I put the cut gel into the weighed sterile Eppendorf tube and weighed it. For every 100 mg of agarose gel, 200 µl buffer NTI was added. The sample was then incubated for 5-10 minutes at 50 °C. The sample was loaded into the Nucleo SpinGel and PCR Clean up Column, centrifuged for 30 seconds at 11000 g and the flow was removed. I added 700 µl NT3 buffer to the column and centrifuged for 30 seconds at 11000 g, then discarded the flow and repeated the washing step. I centrifuged for 1 minute at 11000g and then threw out the flow to remove the buffer NT3. Then I placed the column in a new Eppendorf tube, applied 3x20 µl 70 °C IU buffer, kept it at 70 °C for 5 minutes, then centrifuged at 30-50 g for 1 minute and then at 11000 g for 1 minute.

BIOMI Kft. (Gödöllő, Hungary) sequenced PCR products. The sequences were submitted to the National Library of Medicine at the National Center for Biotechnological Information (NCBI) under the OP183257 - OP183263 registry numbers.

2.5. Mycotoxin resistance in bacteria

For all resistance tests, Biopure mycotoxin solutions (Romer Labs, Tulln, Austria) were used in appropriate dilution. The isolates were inoculated into MRS broth (Scharlab) for LABs and Nutrient broth (Scharlab) for other bacteria and incubated for 16 hours at 30 °C to obtain exponential phase cultures. I inoculated a microtiter plate with 200 µl MRS medium or Nutrient Broth to obtain a low-density culture (0.1-0.2 OD_{630nm}). Mycotoxins were added to the cultures in different concentrations (AFM1: 0-1.47 µg/L; AFB1: 24-50-100 µg/L; OTA: 50-1000 µg/L; DON: 700-1000 µg/L; ZEA: 100-200-500 µg/L). The bacteria were incubated with mycotoxins for 24 hours at 30 °C in a microtiter plate reader (Synergy HTX Multimode Reader, BioTek). The optical density was read hourly at 630 nm after intense shaking (30 sec). The growth curve of untreated cultures (without mycotoxins) was compared with mycotoxin-treated cultures (n = 4). I determined the final percentage from the 24th hour result. The analysis of growth data was processed in Gen5 3.05 software (Bio-Tek) and Microsoft Excel Analísis ToolPac Pearson's t trial ($p \leq 0.05$).

2.6. Preparation of bacterial cell fractions

Pellets produced from bacterial strains were treated with various chemicals (Niderkorn et al; 2009; Goh et al., 2009), during which I received various fractions (Table 1).

Table 1: Various treatments and bacterial preparations created by them (Niderkorn et al., 2009; Goh et al., 2009)

Treatment	Bacterial preparates
H_2O 100 °C, 15 min	cell debris
2% w/v sodium-dodecyl-sulphate (SDS) 100 °C, 15 min	cell wall fraction
10% w/v trichloracetic acid (TCA) 100 °C, 15 min	peptidoglucan (PG) fraction
1 M LiCl 100 °C, 15 min	S-layer fraction
1 M HCl 100 °C, 15 min	teichoic acid fraction

2.7. Test of mycotoxin elimination in bacteria

The mycotoxins (BIOPURE, Romer Labs, Tulln, Austria) were diluted with buffered PBS and added to live bacterial cells and various bacterial preparations in the following concentrations: AFB1-24 μg/l, AFM1-1.47 μg/l, DON-700 μg/l, ZEA-100 μg/l, OTA-1000 μg/l. All mycotoxin-supplemented samples were incubated in PBS for 1 hour at 25 °C by shaking (6 g), then centrifuged (6100 g, 10 minutes, 4 °C), supernatants removed and analysed with HPLC as described in Chapter 2.3.1. All studies were performed in three parallels; positive (without cells or cell wall fractions) and negative (without mycotoxins) controls were used.

2.8. Esterase activity

Based on the method of Castillo et al. (1999), the reaction mixture contained 800 μl of 50 mM Tris-HCl buffer (pH 7.5), 100 μl p-nitrophenyl butyrate (Sigma-Aldrich, Saint Louis, USA) as substrate (8.1 mM in acetone), and 100 μl of lysed (Precellys 24 Homogenizer, Peqlab Biotechnologie Ltd., Erlangen, Germany) and PBS-washed cell debris or heat-treated cell wall fraction samples. Enzyme activity was detected as p-nitrophenol after 10

minutes of incubation at 37 °C (346 nm). Esterase activity was expressed in p-nitrophenyl released per minute.

2.9. Silage making

The chopped wet maize crop was treated with 0.1 v/v% formic acid and mixed with a reference sample of *Fusarium* mycotoxin containing maize (Quality Control Material Deoxynivalenol in corn, Biopure, Quality Control Material Zearalenone in corn, BIOPURE, Romer Labs, Tulln, Austria) yielded a final concentration of 0.7 mg/kg DON and 0.01 mg/kg ZEA. A 16-hour culture of *Lactiplantibacillus plantarum* NCAIM B01074 was used as an inoculated strain. A total of four compositions of silage were prepared: controlled, mycotoxin-contaminated, mycotoxin-contaminated and bacteria-inoculated, and bacteria-inoculated only. I kept the corn plant of different composition (raw ~350 g) in plastic boxes, airtight wrapped, at room temperature for 4 weeks, under constant pressure. At the end of fermentation, dry matter, pH, volatile acid content and mycotoxin content were determined.

2.10. Determination of dry mass

Fermented fodder samples (maize, alfalfa, rye) or maize were dried in three parallel (60 °C±1 °C) in an oven (UN55, Memmert GmbH, Germany). Before and after drying, the weight of the samples was weighed, from which dry mass was calculated (n = 3; CV%, coefficient of variation ≤ 10%). The coefficient of variation is the quotient of the standard deviation and the mean of the values measured in parallel, expressed as a percentage and equal to the relative standard deviation. RSD= 100*S/M, where S is the standard deviation and M is the arithmetic mean. Drying to constant weight (ISO 6496, 1999).

2.11. pH determination

pH measurement of the squeezed juice of the prepared silage samples was done with automatic pH measuring equipment (pH1100L; VWR International Ltd.) (n = 3, CV% ≤ 10%).

2.12. Determination of lactic acid and volatile acid content

The lactic acid content of silage samples was determined by HPLC-DAD method. A sample of 50 g of silage was weighed into a conical flask, sealed with a parafilm after adding 300 ml of water and stored at 4 °C. The sample was filtered using pleated filter paper followed by a 0.45 μm Spartan syringe filter (Merck). For the sample, Phenomenex

(Torrance, CA, USA) RP-C18 column (125*4 mm, 5 μm) was used with DAD detector at UV 218 nm and acetonitrile: water (10:90) eluent.

To determine the volatile acid content, 50 g of silage sample was weighed into a conical flask, sealed with a parafilm after adding 300 ml of 1 v/v phosphoric acid solution and stored overnight at 4 °C. After filtration (pleated filter paper, then 0.45 μm Spartan syringe filter), the following components were determined by gas chromatography, measured by DE MEK Agricultural Instrument Center: acetic acid, propionic acid, i-butyric acid, n-butyric acid, n-butyric acid, i-valerian acid, n-valerian acid, i-caproic acid, n-caproic acid, heptanoic acid. The standard was Fatty Acid Methyl Esters (FAME) mix (Sigma-Aldrich). LOD= 0.002 m/m%, n = 3.

2.13. Statistical analysis

The correlation analysis was performed using Microsoft Excel's data analysis ToolPac. Growth data analyses were performed in Gen5 3.05 software (BioTec) and Microsoft Excel Analysis Tool-Pac, where Pearson t-test ($p \leq 0.05$) was performed for significance analysis.

3. RESULTS

3.1. Characterisation of industrial fermented feed samples

The dry weight of fermented feedingstuffs ranged from 19% to 71%.

The samples mainly measured DON contamination, and the concentration range was quite wide, from 0.100 mg/kg to 3.254 mg/kg. The average contamination by DON in maize silage was 0.901 ± 0.012 mg/kg. Other mycotoxins were detected, and AFB1 and AFG2, and in some cases, ZEA, were measured in fermented feeds. OTA and AF contamination were characteristic of fermented alfalfa products in both silage and haylage samples, and DON was also detected in silage.

Triticale haylage samples were characterised by a relatively high lactic acid bacteria count with low mycotoxin content (2.5-5.3 $\log_{10}$ CFU/g). Microbial counts in fermented feeds varied widely, with logarithmic values ranging from 3.265 to 7.254 $\log_{10}$ colony froming unit (CFU)/g. The logarithmic value of colony numbers from the MRS agar medium ranged from 1.778 to 6.167 $\log_{10}$ CFU/g. The total colony counts of fermented feeds were

closely related to colony counts of MRS agar, which mostly cultured lactic acid bacteria, as the correlation coefficient was 0.848, which showed a strong positive correlation.

Mesophilic sulfite-redulating *Clostridiums* were present in samples variously (1-5.4 $\log_{10}$ CFU/g) and their numbers were not correlated with either total microbial counts or LAB counts. Most samples were characterized by low mould count (1 $\log_{10}$ CFU/g), but some samples contained high mould contamination (3.00 - 6.079 $\log_{10}$ CFU/g). High mould contamination was in no case associated with high mycotoxin content. Lactic acid bacteria had only a very weak negative correlation with mycotoxins (AF: -0.360; OTA: -0.336).

The final total microbial count consisted largely of lactic acid bacteria, and their viable cell count did not depend on the final water content of the feed, as the correlation coefficient was only 0.219 between the moisture content of fermented feed (29-81%) and the viable lactic acid bacteria count.

3.2. Identification of isolated bacteria of fermented feed origin

Bacteria identified with MALDI-TOF MS: *Lactiplantibacillus plantarum* in maize silage, *Lactiplantibacillus pentosus* in alfalfa silage, and *Pediococci* in rye silage and hayage samples. Some of the aerobic spore-forming bacteria (eg. *Lysinibacillus spp.*, *Rummeliibacillus spp.* and *Bacillus spp*.), and an enterobacterium, *Klebsiella pneumoniae*.

In identification using 16S rRNA gene sequences, *Rummeliibacillus suwonensis, Bacillus thuringiensis, Lysinibacillus boronitolans, Lysinibacillus fusiformis.* With the exception of *Rummeliibacillus suwonensis* (*Planococcaceae*), all isolates belonged to the family *Bacillaceae* (Vos et al., 2011; Federhen, 2015; Schoch et al., 2020).

3.3. Mycotoxin resistance of isolated bacteria of fermented feed origin

DON (1000 μg/l) and ZEA (500 μg/l) did not inhibit cell growth, but OTA did inhibit bacterial growth, with 33-86% inhibition observed at 1000 μg/l OTA concentrations. Lower concentrations of mycotoxins did not cause any change in bacterial culture growth, and lactic acid bacteria, spore-forming bacteria and *Klebsiella pneumoniae* were no different in this trait. AFB1 at a concentration of 24 μg/l caused inhibition of growth in some lactic acid bacteria isolates (*L. curvatus, L. brevis, L. coryniformis*), but only up to a maximum of 24% in *L. brevis*. The growth of isolated spore-forming bacteria (*Lysinibacillus spp., Rummeliibacillus suwonensis* and *Bacillus thuringiensis*) and *Klebsiella pneumoniae* up to 100 μg/l was not affected by AFB1.

3.4. Mycotoxin elimination of live cells and cell wall fractions of isolated bacteria of fermented feed origin

For *B. thuringiensis, L. boronitolerans, L. fusiformis, R. suwonensis,* DON elimination was negligible. The AFB1 elimination potential was below 20%, except for the S-layer protein fraction *B. thuringiensis*, where 64% AFB1 elimination occurred (Figure 1).

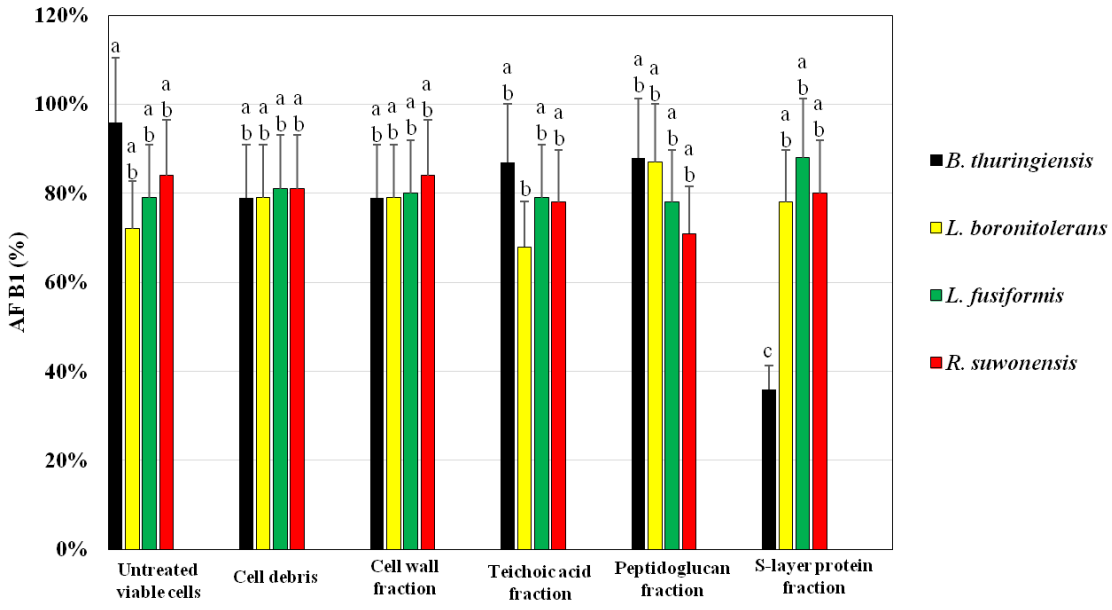


Figure 1. AFB1 (n = 3, CV% ≤ 15%) measured from the supernatant after AFB1 elimination test with viable cells of *Bacillus thuringiensis* AMK10/1, *Lysinibacillus boronitololans* AMK9/1, *Lysinibacillus fusiformis* AMK10/2 and their bacterial preparations. The letters above each column indicate the result of comparing all samples in pairs. The results indicated by the same letter do not differ significantly (t test, p ≤ 0.05).

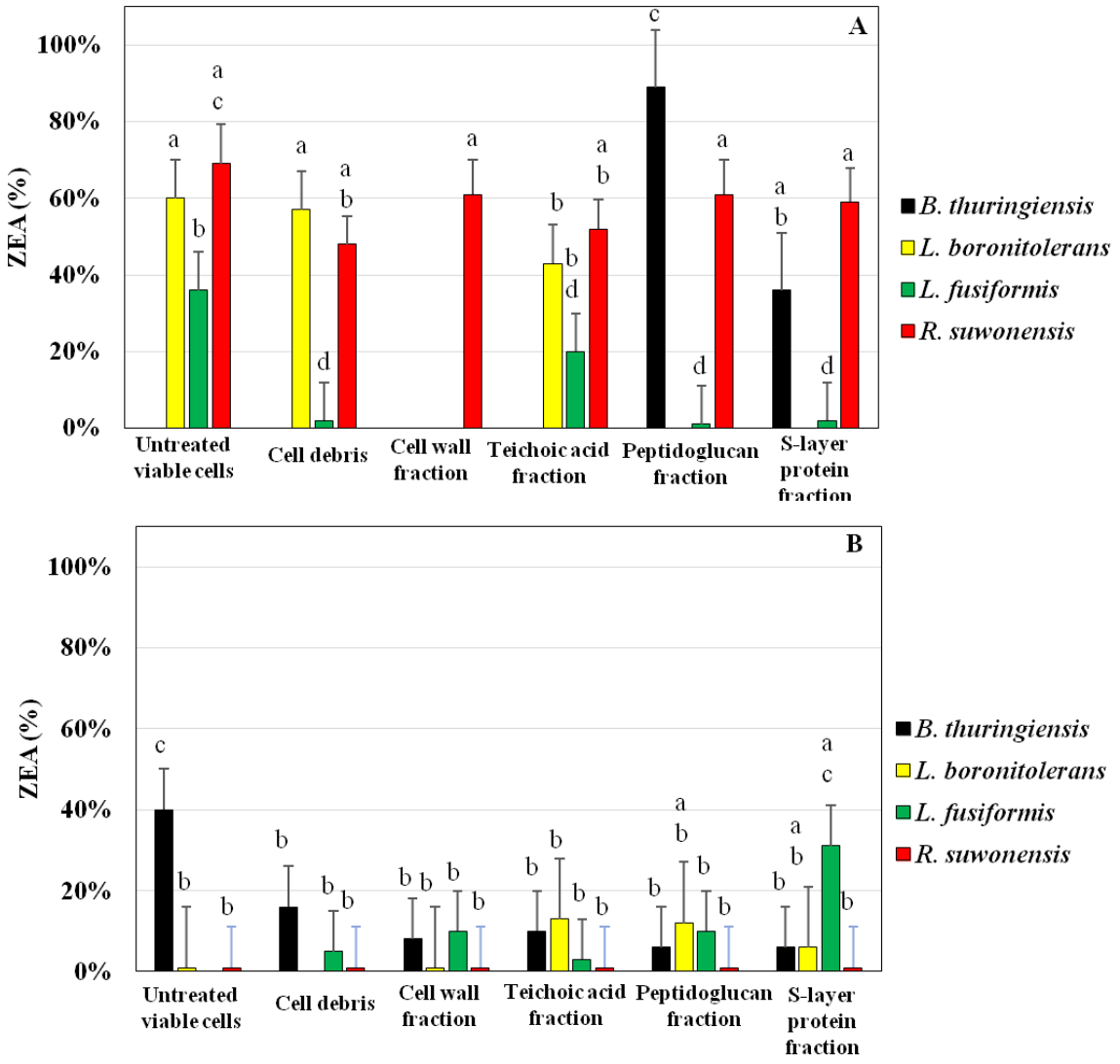


Figure 2. ZEA concentration (n = 3, CV% ≤ 15%) measured from the supernatant (A) and pellets (B) following a ZEA elimination test with *Bacillus thuringiensis* AMK10/1, *Lysinibacillus boronitolerans* AMK9/1, *Lysinibacillus fusiformis* AMK10/2 and *Rummelinibacillus suwonensis* AMK9/2 viable cells and their bacterial preparations. The letters above each column indicate the result of comparing all samples in pairs. Columns marked with the same letter do not differ significantly from each other (t test, $p \leq 0.05$).

Figure 2A shows that the viable cells and its preparations of *Rummeliibacillus suwonensis* AMK9/2 were showned identical levels of ZEA elimination (about 40%). Importantly, the purified cell wall, PG, and S-layer protein fractions of *L. boronitolorans* AMK 9/1 almost completely eliminated ZEA and the remaining ZEA concentrations in supernatants were below LOD. ZEA concentrations extracted from different cell wall fraction pellets ranged

from 1% to 12% of values (cell wall fraction: 1%, PG fraction: 12%, S-layer protein fraction: 6%) (Figure 2A).

In the case of *Lysinibacillus fusiformis* AMK10/2, cell debris, purified cell walls, PG fraction, and S-layer protein fraction showed outstanding results in ZEA elimination (Figure 2A). However, recovery of ZEA from the fractions was only significant in part except for the S-layer protein fraction of *Lysinibacillus fusiformis* AMK10/2, where 25% of the original ZEA was eliminated (Figure 2B).

In the case of *Bacillus thuringiensis* AMK10/1, viable cells, cell debris, purified cell walls, and the teichoic acid fraction were suitable for removing ZEA (Figure 2A). For the PG fraction, ZEA elimination was negligible, while for the S-layer protein fraction it was 38%. ZEA was released from AMK/10/1 cells and cell wall fractions during extraction (Figure 2B).

No DON elimination was observed for *Klebsiella pneumoniae*. AFB1 elimination was significant from purified PG (15%), purified cell wall (18%), teichoic acid (20%), and cell debris (27%) fractions. Based on ZEA elimination studies, it can be concluded that in *Klebsiella pneumoniae* no ZEA toxin was measured from the purified cell wall (0%), S-layer protein (0%) and cell debris (0%) supernatant, but significant amounts of ZEA toxin were detected in purified PG fraction (84%) and teichoic acid fraction (87%). Based on the ZEA concentration measured from the pellets, the toxin was detectable in high amounts in purified cell walls (77%), cell debris (76%), while purified PG (9%), teichoic acid (11%) and S-layer protein (0%), fractions did not contain significant amounts of toxin.

3.5. Esterase activity

R. suwonensis AMK9/2 and *B. thuringiensis* AMK10/1 produced higher esterase activity than *L. fusiformis* AMK10/2 and *L. boronitolerans* AMK9/1. The highest enzyme activity was measured in *R. suwonensis* AMK9/2 cell debris (1.98 ± 0.3 mM p-nitrophenol release/min).

3.6. Aflatoxin M1 resistance and elimination of dairy microorganisms

In *L. lactis ssp. lactis* R703 culture, during its exponential growth phase, a small but significant decrease in cell density was observed at a high AFM1 concentration (1.47 µg/l).

In contaminated milk (AFM1 concentration: 30±5 ng/kg), the PG fraction of *Lactococcus lactis ssp. lactis* R703 eliminated most AFM1 (58%). Unlike the PG fraction, the AFM1

content of milk was high for all fractions, purified cell wall (75%), teichoic acid fraction (84%) and cell debris (91%). However, 67% of the initial concentration of AFM1 remained in milk when untreated, viable cells were used.

With *Bifidobacterium animalis ssp. lactis* BB12 PG fraction, milk had the lowest AFM1 content (60%). Following the PG fraction, the AFM1 content of milk was higher than the other fractions: purified cell wall (78%), teichoic acid fraction (70%), cell debris (75%), viable cells (81%). Different amounts (50, 100, 150 µl) of bacterial PG fractions yielded similar residual AFM1: 60%, 68%, and 62% in milk, with no significant differences. For *Lactococcus lactis ssp. lactis* R703, increased PG fractions (50, 100, 150 µl) resulted in the following residual AFM1 in milk: 58%, 65% and 59% respectively. Based on these, it was concluded that increasing amounts did not increase AFM1 elimination.

AFM1 elimination of lactic acid bacteria *L. paracasei ssp. paracasei* 431 was not significant. 84% of the initial concentration of AFM1 remained in milk for cell fractions treated with SDS and H_2O.

For R703 and BB12 strains, using a two-hour treatment time, AFM1 elimination of the cell preparations did not increase compared to one-hour incubations of the same biomass preparations. For PG fractions of R703 and BB12, AFM1 toxin remained in milk at 78% and 68%, respectively. Untreated BB12 did not remove AFM1 toxin from milk. Application of viable R703 bacteria resulted in 79% of the original AFM1.

For strain BB12, the mycotoxin elimination ability of the PG fraction was better than that of viable cells. For strain R703, there was no significant difference in the elimination ability of living cells and PG fractions. The longer incubation time reduced the amount of AFM1 eliminated in both living cells and PG fractions of R703 and BB12 strains.

3.7. Mycotoxin elimination in an experimental silo

The lowest pH value was shown in the control sample (pH 4.55). The pH of the mycotoxin-contaminated sample was 4.57. The sample contaminated with mycotoxin and inoculated with *Lactiplantibacillus plantarum* NCAIM B01074 culture showed pH 5.16, and the pH of the sample inoculated with NCAIM B01074 culture only was 5.01. The initial dry matter content was 19.90%.

The lactic acid content did not differ significantly from the control sample in the mycotoxin-containing sample at the end of the process, while it was lowest in the silo sample treated with only *L. plantarum* NCAIM B01074 cells. The acetic acid content in

the control sample was high, while in the other *in vitro* ready silage it showed low values. The smallest amount of propionic acid was detected in the silage sample containing the cell. Low concentrations of isobutyric acid were present in all four treatments. N-butyric acid was present at the lowest concentration in the control silage sample. Isovalerian acid was present in the control sample at higher concentrations than in the other silage samples. For n-valerian acid, it was detectable only in the silage sample contaminated with toxins, while it was present in the other samples at concentrations below the lower limit of measurement. Isocaproic acid was detected in the control sample, while it was below the measurement limit in the other three samples. Heptanoic acid was below the measuring limit (LOD= 0.002 m/m%) for all four silage samples.

In the case of the control sample, DON mycotoxin could not be measured in the shredded maize crop, but it could be detected in the finished silage. In a sample contaminated with mycotoxin and inoculated with cells, a significant decrease in toxin content can be observed. In a sample inoculated with *L. plantarum* NCAIM B01074, no DON was measured, neither in the shredded maize crop nor in the finished silage. ZEA toxin was detected in the control chopped maize plant. With the same amount of ZEA toxin, fewer samples inoculated with mycotoxins and cells were detected. However, none of the finished silage samples showed ZEA toxin.

4. NEW SCIENTIFIC ACHIEVEMENTS OF THE THESIS

1. The number of final total LABs does not depend on the final water content in the fermented feed (Pearsons correlation coefficient: 0.219).
2. Ochratoxin A inhibits (33-86%) the growth of the LAB strains I studied.
3. Mycotoxin resistance and elimination ability of the bacterium *Rummeliibacillus suwonensis* were investigated first time. For *R. suwonensis*, the ability to eliminate deoxynivalenol was negligible. The elimination potential of aflatoxin B1 was below 20%. Cells and cell wall fractions removed zearalenone in equal proportions (40%).
4. The mycotoxin resistance and elimination ability of the bacterium *Lysinibacillus boronitolerans* were investigated first time. The strain ability to eliminate deoxynivalenol was negligible. The elimination potential of aflatoxin B1 was below 20%. The purified cell wall, PG, and S-layer protein fractions eliminated zearalenone. The remaining ZEA concentrations in the supernatants were below the LOD (2.6 μg/l).
5. It was found that the S-layer protein fraction (64%) of *Bacillus thuringiensis* eliminates aflatoxin B1 toxin at higher levels than other cell wall fractions or whole cells (20%).
6. It was established that the gram-negative bacterium *Klebsiella pneumoniae* is capable of elimination of zearalenone. The initial zearalenone was not detectable on purified cell wall (0%), S-layer protein (0%), and cell debris (0%) from the supernatant.
7. It was found that the elimination of the PG fraction (40%) of *Bifidobacterium animalis* ssp. *lactis* BB12 dairy microbe is better than that of the whole cell (19%).
8. AFM1 elimination of *L. paracasei ssp. paracasei* 431 dairy lactic acid bacteria was not significant. Untreated live bacteria not eliminate AFM1. 84% of the initial concentration of AFM1 remained in milk for the purified cell wall and cell debris fractions.

5. PRACTICAL APPLICABILITY OF RESULTS

1. Regardless of how dry the silage feed, large amounts of LAB survive in fermented feed, this probiotic nature makes it suitable for livestock. Low water content, on the other hand, can affect (besides lactic acid) the ability of other organisms to reproduce. This can be exploited to maintain a high cell count.
2. The relatively low number of LAB species was characteristic of the last stage of fermentation. From the bacteria present in this stage (i.e. acid-tolerant and mycotoxin-resistant), I identified and characterized some non-lactic acid bacteria with unknown mycotoxin elimination potential. Their activity can inhibit fungal growth and mycotoxin contamination under aerobic starting conditions of ensiling or after silage openings.
3. In a silage experiment, a significant reduction in mycotoxin content was observed in a sample contaminated with mycotoxin and inoculated with *Lactiplantibacillus plantarum* NCAIM B01074. In thea sample inoculated with *L. plantarum* NCAIM B01074, no DON was measured, neither in the shredded maize crop nor in the finished silage. However, none of the finished silage samples showed ZEA toxin. For DON, in addition to the original 42% reduction, *L. plantarum* NCAIM B01074 gave a 75.6% DON reduction taking into account the release of "masked" mycotoxin.
4. The PG cell wall fractions of R703 and BB12 bound significant amounts of AFM1 from naturally contaminated milk over an hour of treatment. Furthermore, the PG fraction was better absorbent for AFM1 than viable cells for BB12, while the difference was insignificant for strain R703. It can be used in dairy industry.

6. BIBLIOGRAPHY

Adácsi C. - Kovács S. - Pócsi I. - Győri Z. - Dombrádi Z. - Pusztahelyi T.: 2022a. Microbiological and toxicological evaluation of fermented forages. *Agriculture*, 12. 421.

Adácsi C. - Kovács S. - Pócsi I. - Pusztahelyi T.: 2022b. Elimination of deoxynivalenol, aflatoxin B1, and zearalenone by Gram-positive microbes (Firmicutes). *Toxins*, 14. 591.

Castillo I. - Requena T. - De Palencia P. F. - Fontecha J. - Gobbetti M.: 1999. Isolation and characterization of an intracellular esterase from *Lactiplantibacillus casei subsp. casei* IFPL731. *Journal of Applied Microbiology*, 86. 653-659.

Federhen S.: 2015. Type material in the NCBI Taxonomy Database. *Nucleic Acids Research*, 43. D1086-D1098.

Goh Y. J. - Azcárate-Peril M. A. - O'Flaherty S. - Durmaz E. - Valence F. - Jardin J. - Lortal S. - Klaenhammer T. R.: 2009. Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactiplantibacillus acidophilus* NCFM. *Applied and Environmental Microbiology*, 75. 3093-3105.

ISO 6496 (1999); Animal Feeding Stuffs—Determination of Moisture and Other Volatile Matter Content. International Standard Organization: Geneva, Switzerland, 1999.

Niderkorn V. - Morgavi D. P. - Aboab B. - Lemaire M. - Boudra H.: 2009. Cell wall component and mycotoxin moieties involved in the binding of fumonisin B1 and B2 by lactic acid bacteria. *Journal of Applied Microbiology*, 106. 977-985.

Pradhan P. - Tamang J. P.: 2019. Phenotypic and genotypic identification of bacteria isolated from traditionally prepared dry starters of the Eastern Himalayas. *Frontiers in Microbiology*, 10. 2526.

Schoch C. L. - Ciufo S. - Domrachev M. - Hotton C. L. - Kannan S. - Khovanskaya R. - Leipe D. - Mcveigh R. - O'Neill K. - Robbertse B. - Sharma S. -Soussov V. - Sullivan J. P. - Sun L. - Turner S. - Karsch-Mizrachi I.: 2020. NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database (Oxford). baaa062. 2020.

Vos P. - Garrity G. - Jones D. - Krieg N. R. - Ludwig W. - Rainey F. A. - Schleifer K. H. - Whitman W. B. (szerk.): 2011. Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes (Vol. 3). Springer Science & Business Media.

7. PUBLICATIONS OF THE CANDIDATE

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/28/2024.PL
Subject: PhD Publication List

Candidate: Cintia Adácsi
Doctoral School: Doctoral School of Nutrition and Food Sciences
MTMT ID: 10075110

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (1)

1. **Adácsi, C.**, Kovács, S., Pusztahelyi, T.: Aflatoxin M1 binding by probiotic bacterial cells and cell fractions.
   Acta Aliment. 52 (4), 579-588, 2023. ISSN: 0139-3006.
   DOI: http://dx.doi.org/10.1556/066.2023.00139
   IF: 1.1 (2022)

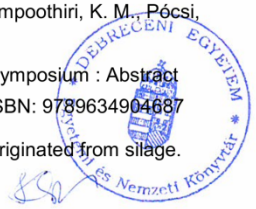
Foreign language scientific articles in international journals (2)

2. **Adácsi, C.**, Kovács, S., Pócsi, I., Pusztahelyi, T.: Elimination of Deoxynivalenol, Aflatoxin B1, and Zearalenone by Gram-Positive Microbes (Firmicutes).
   Toxins. 14 (9), 1-12, 2022. EISSN: 2072-6651.
   DOI: http://dx.doi.org/10.3390/toxins14090591
   IF: 4.2

3. **Adácsi, C.**, Kovács, S., Pócsi, I., Győri, Z., Dombrádi, Z. R., Pusztahelyi, T.: Microbiological and Toxicological Evaluation of Fermented Forages.
   Agriculture-Basel. 12 (3), 1-11, 2022. EISSN: 2077-0472.
   DOI: http://dx.doi.org/10.3390/agriculture12030421
   IF: 3.6

Foreign language abstracts (5)

4. Pusztahelyi, T., Kovács, S., **Adácsi, C.**, Miklós, I., Dobos, A., Győri, Z., Nampoothiri, K. M., Pócsi, I.: Biological control and elimination of mycotoxins in food and feed.
   In: Biotechnology at the University of Debrecen - 2022 International Symposium : Abstract Book, Debreceni Egyetem, Printart-Press Kft., Debrecen, 17, 2022. ISBN: 9789634904687



5. **Adácsi, C.**, Pusztahelyi, T.: Detoxification potential of lactic acid bacteria originated from silage.
   Acta Microbiol. Immunol. Hung. 68, 4, 2021. ISSN: 1217-8950.

6. **Adácsi, C.**, Pusztahelyi, T., Kovács, S.: Mycotoxin resistance and elimination capability of Klebsiella pneumoniae.
   Acta Microbiol. Immunol. Hung. 68 (Suppl.1), 53, 2021. ISSN: 1217-8950.

7. Pusztahelyi, T., **Adácsi, C.**: Non-lactic acid bacteria for biological control of mycotoxin contamination in commodities.
   Acta Microbiol. Immunol. Hung. 68 (Supplement-1), 107, 2021. ISSN: 1217-8950.

8. **Adácsi, C.**, Pusztahelyi, T.: Investigation of Aflatoxin M1 Binding Capacity of Probiotic Bacterial Cultures And Their Preparations.
   In: 4th National Conference of Young Biotechnologists : Abstract Book "FIBOK 2020". Ed.: Tünde Pusztahelyi, Levente Czeglédi, Éva Domokos-Szabolcsy, Tamás Emri, University of Debrecen, Debrecen, 72-72, 2020. ISBN: 9789634902720

List of other publications

Foreign language scientific articles in international journals (3)

9. Molnár, K., Rácz, C., Dövényi-Nagy, T., Bakó, K. I., Pusztahelyi, T., Kovács, S., **Adácsi, C.**, Pócsi, I., Dobos, A.: The effect of environmental factors on mould counts and AFB1 toxin production by Aspergillus flavus in maize.
   Toxins. 15 (3), 1-18, 2023. EISSN: 2072-6651.
   DOI: http://dx.doi.org/10.3390/toxins15030227
   IF: 4.2 (2022)

10. Horváth, E., Pusztahelyi, T., **Adácsi, C.**, Tanyi, E., Pócsi, I.: Optimization and Validation of ELISA for Aflatoxin B1 Detection in Fermented Forages and Feeds.
    Scientifica. 2022, 1-6, 2022. EISSN: 2090-908X.
    DOI: http://dx.doi.org/10.1155/2022/6059880
    IF: 3.2

11. Sipos, P., Horváth, M., **Adácsi, C.**, Horváth, B., Babka, B., Győri, Z.: Enrichment of pasta products using beetroot.
    Food and Environment Safety. 16 (4), 209-215, 2017. ISSN: 2068-6609.

Foreign language abstracts (4)

12. Pusztahelyi, T., Kovács, S., **Adácsi, C.**, Miklós, I., Pócsi, I.: Biological elimination of Fusarium mycotoxins: yeasts and bacteria in work.
    In: International Conference on New Horizons in Biotechnology: Abstract book, CSIR - National Institute For Interdisciplinary Science and Technology (NIIST), Trivandrum, India, 144, 2023.

13. Pusztahelyi, T., **Adácsi, C.**, Kovács, S., Dobos, A., Pfliegler, V. P., Nampoothiri, K. M., Pócsi, I.: Micotoxins in food chain-climate effect and elimination studies.
    Acta Microbiol. Immunol. Hung. 70 (Suppl.), 77-78, 2023. ISSN: 1217-8950.

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

14. Pócsi, I., Murvai, K., Horváth, E., Bodnár, V., Mondok, Á., **Adácsi, C.**, Molnár, K., Dobos, A., Leiter, É., Pfliegler, V. P., Pusztahelyi, T.: Recent Results in Aflatoxin Research at the University of Debrecen.
In: International Conference on New Horizons in Biotechnology: Abstract book, CSIR - National Institute For Interdisciplinary Science and Technology (NIIST), Trivandrum, India, 110, 2023.

15. **Adácsi, C.**, Kovács, S., Miklós, I., Pócsi, I., Pusztahelyi, T.: Resistance of yeasts against Fusarium mycotoxins.
In: "FIBOK 2022" : Fiatal Biotechnológusok V. Országos Konferenciája = 5th National Conference of Young Biotechnologists : Program és angol nyelvű összefoglalók = Program and abstracts. Szerk.: Bánfalvi Zsófia, Gócza Elen, Olasz Ferenc, Pál Magda, Posta Katalin, Várallyay Éva, MATE Genetika és Biotechnológia Intézet, Gödöllő, 84-84, 2022. ISBN: 9789632699998

Total IF of journals (all publications): 16,3
Total IF of journals (publications related to the dissertation): 8,9

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 January, 2024

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *