Food Research International 186 (2024) 114364

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

A new physical and biological strategy to reduce the content of zearalenone in infected wheat kernels: the effect of cold needle perforation, microorganisms, and purified enzyme

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ARTICLE INFO

Keywords:
Mycotoxins
Zearalenone
Wheat
Wheat kernels
Cold needle perforation
Biological decontamination
Bacillus megaterium
Bacillus licheniformis
Enzymatic degradation
Zearalenone hydrolase
HPLC-MS/MS

ABSTRACT

With the aim of reintroducing wheat grains naturally contaminated with mycotoxins into the food value chain, a decontamination strategy was developed in this study. For this purpose, in a first step, the whole wheat kernels were pre-treated using cold needle perforation. The pore size was evaluated by scanning electron microscopy and the accessibility of enzymes and microorganisms determined using fluorescent markers in the size range of enzymes (5 nm) and microorganisms (10 μm), and fluorescent microscopy. The perforated wheat grains, as well as non-perforated grains as controls, were then incubated with selected microorganisms (*Bacillus megaterium* Myk145 and *B. licheniformis* MA572) or with the enzyme ZHD518. The two bacilli strains were not able to significantly reduce the amount of zearalenone (ZEA), neither in the perforated nor in the non-perforated wheat kernels in comparison with the controls. In contrast, the enzyme ZHD518 significantly reduced the initial concentration of ZEA in the perforated and non-perforated wheat kernels in comparison with controls. Moreover, *in vitro* incubation of ZHD518 with ZEA showed the presence of two non-estrogenic degradation products of ZEA: hydrolysed zearalenone (HZEA) and decarboxylated hydrolysed ZEA (DHZEA). In addition, the physical pre-treatment led to a reduction in detectable mycotoxin contents in a subset of samples. Overall, this study emphasizes the promising potential of combining physical pre-treatment approaches with biological decontamination solutions in order to address the associated problem of mycotoxin contamination and food waste reduction.

1. Introduction

Mycotoxins are low molecular weight natural compounds, mainly produced by filamentous fungi of *Penicillium*, *Aspergillus* and *Fusarium* genus. More than 25 % of the crops harvested are contaminated with at least one mycotoxin (Eskola et al., 2019). The infection with mycotoxin producing moulds can happen in the field during plant growth and after the harvest during storage and processing. The contamination of crops with mycotoxins leads to substantial economic losses and food waste, and poses a threat to animal and human health due to both acute intoxication and chronic diseases resulting from long-term exposure, even at doses slightly above the regulation limits.

Zearalenone (ZEA) is a non-steroidal estrogenic compound produced by fungi from the *Fusarium* genus (Wu & Munkvold, 2008). The compound is one of the most prevalent mycotoxins worldwide (Gruber-Dorninger et al., 2019) infecting corn and small grains such as wheat, rice, barley, soybean, oat and millet (EFSA, 2011). The chemical structure of ZEA resembles natural oestrogens, such as 17 β-estradiol. Thus,

Abbreviations: DHZEA, decarboxylated hydrolysed zearalenone; DON, deoxynivalenol; eGFP, enhanced green fluorescent protein; HPLC-MS/MS, High Performance Liquid Chromatography-Tandem Mass Spectrometry; HZEA, hydrolysed zearalenone; ZEA, zearalenone; ZHD, zearalenone hydrolase.

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https://doi.org/10.1016/j.foodres.2024.114364
Received 22 December 2023; Received in revised form 5 April 2024; Accepted 17 April 2024
Available online 19 April 2024

ZEA can bind competitively to oestrogen receptors and other steroid receptors (Molina-Molina et al., 2014), disturb the hormonal balance in mammals and cause reproductive problems. Additional adverse health effects reported in the literature are hepatotoxicity, immunotoxicity, genotoxicity, carcinogenicity, negative effects on gastrointestinal health and endocrine disruptive effects (Pierzgalski et al., 2021).

European authorities have restricted the maximum levels of ZEA allowed in foodstuffs to 100 μg/kg in unprocessed cereals other than maize, 75 μg/kg for cereals intended for direct human consumption and 20 μg/kg for processed cereal-based foods and for foods intended for children and babies (European Commission, 2023). Nevertheless, several studies have shown that various types of agricultural and food products exceed the limits set up by the European authorities for mycotoxins in general, and ZEA in particular (André et al., 2022; Luo et al., 2021; Orlando et al., 2019; Stanciu et al., 2017; Vogelgsang et al., 2017). Moreover, feeding animals with contaminated feed poses the risk of carry-over from food of plant origin such as malt, flour, oil to food of animal origin such as eggs, milk or meat (Dänicke & Winkler, 2015; Döll et al., 2003; Olsen et al., 1986). Considering the mean level of ZEA contamination in the most consumed food, the estimation of the average daily intake of ZEA for adults ranged from 0.8 to 29 ng/kg body weight and for small children from 6 to 55 ng/kg (Han et al., 2022; Minervini et al., 2005). Those values are below the current tolerable daily intake (TDI) of 0.25 μg/kg body weight established by EFSA in 2011 based on oestrogenicity in pigs (European Commission, 2016).

The decontamination of ZEA contaminated grains is difficult as this mycotoxin is rather stable (Krska et al., 2003; Rogowska et al., 2019). In the cereal industry, even if milling and separation into pure white flour and bran fractions have been shown to reduce the microbial contamination (Laca et al., 2006), mycotoxins are not totally removed during processing steps and can be found therefore in the final feed or food product. Contaminated corn has for example been used for production of bioethanol (Bennett et al., 1981). Yet, while the produced ethanol was free of mycotoxin, in co-products like distillers dried grain with solubles (so-called DDGS) used broadly in the United States to feed livestock, the mycotoxin content was found to be up to three times higher than in the starting material (Makkar, 2014; Wu & Munkvold, 2008). Moreover, physical or chemical decontamination methods have a negative influence on quality of the feed or food by removing for example valuable dietary components from the raw material. Physical and chemical decontamination methods affect the nutritional value of the raw material, change its color and flavor, induce changes in starch structure, lipid oxidation and fatty acid profile, cause protein denaturation or changes in processing properties (for example gelatinization properties) (Han et al., 2022; Liu et al., 2022). In addition, during treatment, secondary contamination by the chemicals used for decontamination can occur, and metabolites of concern to human and animal health might be produced.

At the same time, food waste is a topic of concern worldwide as approximately 31% of all food produced ends up as waste along the food value chain either during or after agriculture production, storage and food processing or in retail, gastronomy or households (FAO, 2019). As contamination cannot be avoided completely and current decontamination strategies are not totally effective, new approaches are urgently needed to allow making use of this wasted food material.

An emerging strategy is the so-called biological decontamination using microorganisms or enzymes, to bind or biotransform mycotoxins. Microorganisms such as bacilli or lactic acid bacteria are known to reduce the amount of different mycotoxins (Guan et al., 2023; Hassan et al., 2021; Muhialdin et al., 2020) and in particular of ZEA (Chen et al., 2019; Liu et al., 2022; Liu et al., 2023; Średnicka et al., 2021). Their mechanisms of action are diverse, the toxin can either bind to the cell wall or to cell proteins, or the microorganisms have the capacity to degrade the toxins enzymatically (Sadiq et al., 2019).

The enzymatic degradation of ZEA by zearalenone hydrolase was first described in 2002 (Kakeya et al., 2002; Takahashi-Ando et al., 2002). The enzyme ZHD101 was shown to cleave ZEA and form the degradation product HZEA (hydrolysed zearalenone) followed by the spontaneous formation of a second product DHZEA (decarboxylated-hydrolysed zearalenone) (Takahashi-Ando et al., 2002; Vekiru et al., 2016). Both degradation products exhibit markedly reduced estrogenic activity *in-vitro*, as well as *in-vivo* (Fruhauf et al., 2019; Kakeya et al., 2002) and were therefore declared of no toxicological concern by EFSA (EFSA Panel on Additives, Products or Substances used in Animal Feed (FEEDAP) et al., 2022).

The original enzyme ZHD101 has an optimal activity around 40°C and pH 10.5 (Takahashi-Ando et al., 2004). Meanwhile several enzyme variants have been described in the literature (Fang et al., 2022). An enzyme of interest is the enzyme ZHD518 which similar thermostability than the original variant but a maximum activity at pH 8.0 (Wang et al., 2018; Zheng et al., 2018).

Few applications of these biological decontamination strategies to contaminated cereals, feed or food items are reported in the literature. Examples are found for the enzymatic degradation of ZEA in artificially infected wheat grain (Shcherbakova et al., 2020), naturally contaminated corn flour (Ji et al., 2022), corn oil (Chang et al., 2020) and artificially contaminated animal feed (Gruber-Dorninger et al., 2021). In the study by Gruber-Dorninger et al., the disappearance of ZEA in the feed and the production of the non-estrogenic degradation products HZEA and DHZEA was shown. However, applications on naturally contaminated wheat grains have not yet been reported.

This study presents a strategy for decontaminating naturally contaminated wheat grains, with the ultimate aim of reintroducing them into the feed and food value chains. The strategy consists of a pre-treatment step of the whole wheat kernels using cold needle perforation to ease the access of active substances into the wheat kernel followed by the application of the biodetoxification treatments consisting of one of two microorganisms or an enzyme. The goal was to investigate the effect of the kernel pre-treatment on the decontamination efficiency on one hand, as well as to compare the effect of the microbial and enzymatic treatments on the degradation of the mycotoxin zearalenone on the other.

2. Materials and Methods

2.1. Samples, chemicals and reagents

Wheat samples naturally contaminated with ZEA (origin Romania, harvest 2019) were stored at room temperature in the dark in closed containers (moisture content 8.5 %). All solvents and chemicals were purchased from commercial suppliers (VWR, Sigma and Carl Roth) and were used without further purification.

ZEA-free wheat grains (verified using the analytical method described in paragraph 2.9.3) were purchased from Zwicky (Whole Wheat Cleaned, Müllheim-Wigoltingen, Switzerland) and used as the blank sample and as matrix for the matrix-matched calibration described in paragraph 2.9.4.

A pure standard of Zearalenone was obtained commercially (Sigma-Aldrich Merck AG, Zug, Switzerland). All solvents and mobile phase modifiers were of LC-MS grade. Acetonitrile and methanol were supplied by Sigma-Aldrich (Merck AG, Zug, Switzerland). LC-MS grade water was supplied by Carl Roth AG (Arlesheim, Switzerland). LC-MS grade formic acid was supplied by VWR International GmbH (Dietikon, Switzerland).

2.2. Plasmid construction and protein expression

Gene *zhd518* was synthesized by Twist BioSciences (USA) based on XM_013418296 with a N-terminal His-Tag (MGSSHHHHHH SSGLVPRGSHM) and cloned into pET28b(+) using the Nco1 and Xho1 restriction sites. The plasmid constructed was transformed into chemically competent *E. coli* BL21 (DE3) by heat shock treatment. For protein

expression, a single colony was used to inoculate 5 mL of Luria-Bertani (LB) medium containing 50 μg/mL kanamycin) and incubated overnight at 37 °C, 200 rpm. For overexpression, 2 mL of the overnight culture (LB medium with 50 μg/mL kanamycin) were used to inoculate 200 mL ZYM-5052 autoinduction medium (Studier, 2005) and the culture incubated at 25 °C, 160 rpm for 18 h. The cells were harvested by centrifugation at 3700 rcf, 4 °C for 30 minutes.

For protein purification, the cell pellet was dissolved in lysis buffer (50 mM NaP pH 8.0, 0.01 mg/mL DNAse, 0.5 mg/mL Polymixin B, 1.0 mg/mL lysozyme) and cells lysed by sonication (3x1min with 2-sec intervals, 50% amplitude) on ice. The cell suspension was then centrifuged for 30 min at 3700 rcf at 4 °C and the resulting supernatant was filtered using a 0.45 μm filter and applied to a HisTrap HP 5 mL (GE Healthcare, Massachusetts, USA) column that had been pre-equilibrated in binding buffer (25 mM Imidazol, 50 mM NaP pH 8.0). The protein was eluted using a linear gradient from 0 % to 100 % elution buffer (50 mM NaP pH 8.0, 500 mM imidazole). Collected fractions containing the protein were combined and desalted with a HiTrap desalting column (5 mL x 3) equilibrated with 50 mM NaP, pH 8.0. The concentration of the enzyme was determined using a NanoDrop spectrometer (Thermo Fisher Scientific) at 280 nm and the estimated extinction coefficient of 46,870 M^{-1} cm^{-1}. The purity of the proteins was checked by SDS-PAGE analysis (Supplementary Material, Fig. S1). Protein aliquots of 300 μL (2 mg/mL enzyme, 0.1% glycerine added) were flash-frozen in liquid nitrogen and stored at -80 °C.

2.3. Enzymatic assay and determination of enzymatic degradation products of Zearalenone

Enzymatic activity was determined in 50mM NaP buffer pH 8.0 at 40°C containing 50 μg/mL Zearalenone and 60 μg/ml enzyme ZHD518. The reaction mix (0.5 mL) was incubated for 30 minutes, 3 hours, 24 hours and 48 hours in a heating block at 600 rpm. Reaction was stopped by diluting the reaction mix 1:1 with MeOH and the solutions kept frozen until further analysis.

HPLC-MS/MS analysis

LC–MS/MS analyses were conducted on a system consisting of an Agilent 1290 Infinity II chromatographic system coupled to an Agilent 6530 Q-TOF mass spectrometer.

Separation of analytes was performed using an Agilent Poroshell 120 EC-C18 (2.1 × 100 mm, 2.7 μm) column protected by a guard column (Agilent EC-18, 2.1 × 5 mm, 2.7 μm) (Agilent Technologies, Basel, Switzerland).

For ZEA analysis, the flow rate was set to 0.28 mL/min, and the column temperature was set at 35 °C. The two elution mobile phases were made up of water + 0.1% acetic acid (mobile phase A) and methanol + 0.1% acetic acid (mobile phase B). Gradient elution was as follows: 0–0.5 min, 10% B; 6–15 min, 98% B; 15.10–17 min, 10% B. Re-equilibration time was 6 min. Injection volume was 1 μL. The MS analyses were performed using an Agilent 6530 Q-TOF instrument in negative ionization mode (ESI–) in the spectral range of 100–700 Da. Nitrogen served as the nebulizer and collision gas. The MS parameters were as follows: gas temperature, 350 °C; drying gas, 10 L/min; nebulizer, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 3500 V (for ZEA); fragmentor voltage, 100 V. The collision energy for the MS/MS experiments was set at 20 eV.

The data analysis was performed using Mass Hunter Qualitative Analysis Software (v 10.0, Agilent). The degradation products of zearalenone were identified based on their MS/MS spectra according to literature.

2.4. Preparation of grains

The grains were prepared according to the methods previously described by (Stäheli et al., 2023).

Wheat kernels were perforated using a cold needle perforation prototype machine consisting of 3'120 needles with a tooth-like geometry (Fig. 1A). The gap width (gap between perforation unit and conveyor belt) was set to 0 mm and the speed was adjusted to a throughput of 6 kg/h. All kernels were passed through the perforation equipment 10 times to achieve an even distribution of elongated perforations in the surface of the kernel as shown in scanning electron microscope images (Fig. 1B).

2.5. Determination of grain accessibility for enzymes and microorganisms

Non-perforated and perforated wheat kernels were soaked in 98 % ethanol solution containing AlexaFluor 488 (concentration 4.97 x 10^{14} beads per mL, green fluorescing) for 4 h at 6°C to mimic enzymes with a size of about 5 nm (e.g. ZHD 518 used here), then dried at 25°C for 4 hours before being soaked in water containing Nile Blue melamine resin beads (concentration 2.23 x 10^{6} beads per mL, red fluorescing) for 4 h at 6°C to mimic microorganisms with a size of about 10 μm. The kernels were then dried with a paper towel before being cast in a wax mould at 70°C and cooled to room temperature. 10 μm slices were then cut off from the wax mould using a microtome (Rotationsmikrotom CUT 4062, SLEE medical GmbH, Nieder-Olm, Germany). Thereafter, the kernels were visually assessed according to the protocol described by Stäheli et al. (2022) using a fluorescence microscope (ECHO, Revolve, San Diego, United States of America) with the filter sets DAPI (excitation (EX): 385/30, emission (EM): 450/50, dichromatic mirror (DM): 425), FITC (EX: 470/40, EM: 525/50, DM: 495) and TRITC (EX: 530/40, EM: 590/50, DM: 560).

2.6. Determination of the optimal wetting volume

To determine the minimal volume needed for the wetting of the kernel, control solutions with eGFP were produced, that according to Stäheli et al. (2022) mimic the enzyme ZHD518 in terms of molecular size and thus provide insight into diffusion mechanisms. Concretely, perforated samples were wetted by adding 2%, 5% or 10% (*w/w*) of solution containing 12 μg/mL of an enhanced green fluorescent protein eGFP (Gene and Cell Technologies, eGFP, Richmond, United States of America) and then stored for 24 h at 37 °C. Thereafter, the kernels were visually assessed using a fluorescence microscope according to the protocol described previously. The moisture content was also determined using a halogen moisture analyzer (Mettler Toledo, HR83, Columbus, United States of America) with a drying temperature of 180 °C after granulating the wet wheat kernels in a mixer (Vorwerk, Thermomix TM6, Wuppertal, Germany).

2.7. Treatment of grains with microorganisms

The two microorganisms *Bacillus megaterium* Myk145 and *B. licheniformis* MA572 (Mischler et al., 2024) were grown overnight in BHI broth. The treatment of the contaminated grains with microorganisms was performed as follow: 25 g of perforated or non-perforated grains was weighed into 50 mL Falcon tubes and 4.7 mL of sterile water was added, followed by 0.3 mL of a culture suspension of Myk145 or MA572 containing 5.0 x 10^{7} cells per mL (end concentration of the two microorganisms: 5.0 x 10^{5} cells per g grains). For blank controls, the culture suspension was replaced by adding 0.3 mL of the dilution solution to the grains. Each Falcon tube was homogenized by shaking. All tubes were incubated for 72 hours at 30°C. The assay was made in a tenfold repetition (Fig. 2).

To verify the growth of the microorganisms, cell counting was done by taking 3 samples from timepoint zero and 5 samples after 72 hours incubation, from each treatment and control, and analyzed by microbial cultural method onto PC agar plates incubated for 24 hours at 30°C. Some colonies from the plates were taken for analysis by Matrix-Assisted Laser Desorption-Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) to identify the species grown during the 72 hours of incubation.

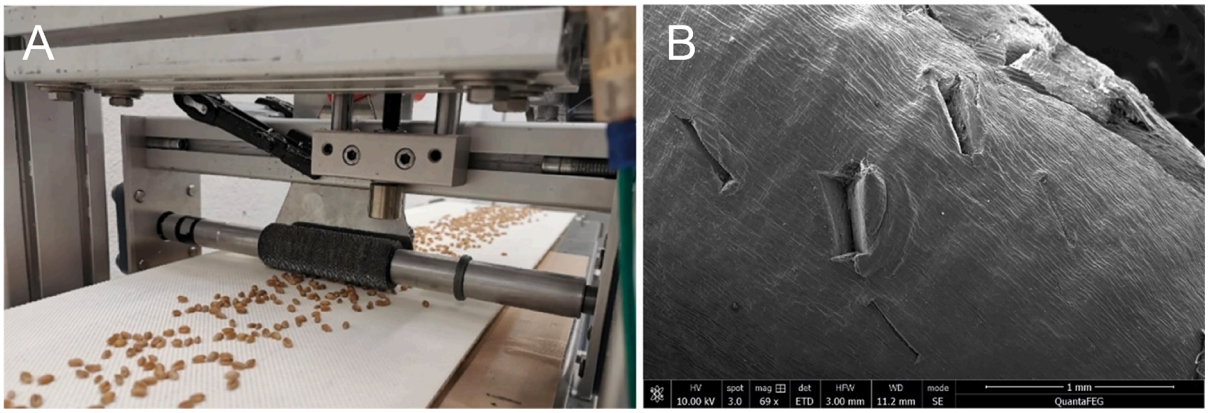

Fig. 1. (A). Cold needle perforation prototype, conveyor belt and needle wheel; (B). Exemplary scanning electron microscope image of typical elongated perforations achieved by cold needle perforation on the wheat kernel surface.

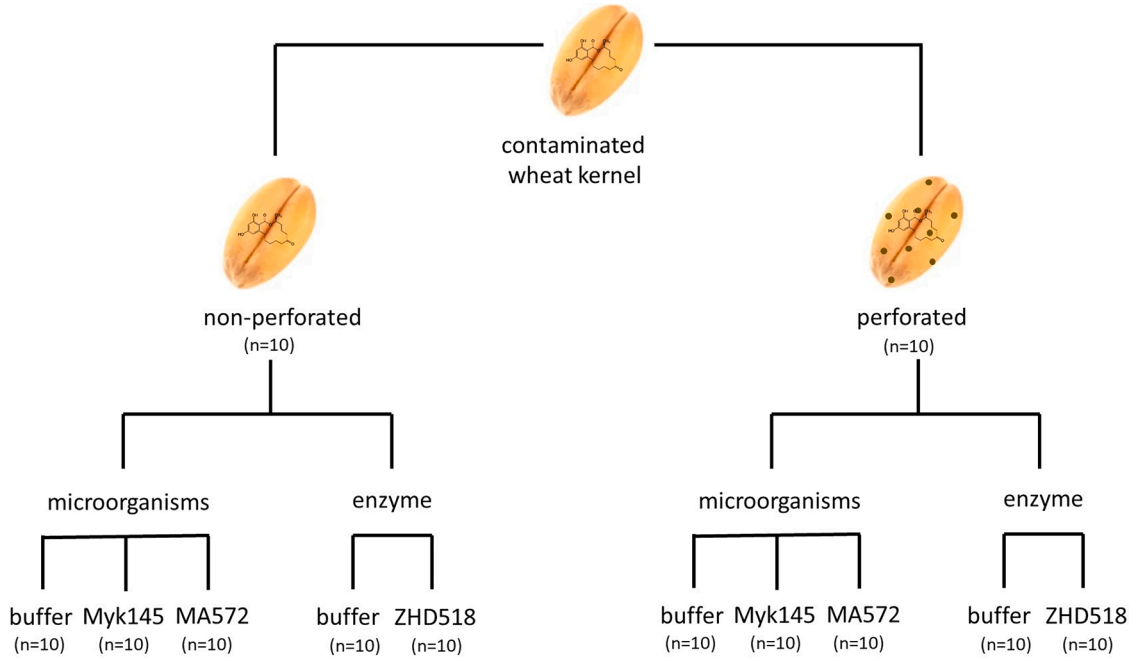

Fig. 2. Experimental set-up: non-perforated as well as perforated kernels were analyzed for content of ZEA before (blank) and after treatment with either buffer (control), microorganisms (Myk145 or MA572) or enzyme (ZHD518).

For toxin determination the samples were frozen at -20°C after the 72 hours of incubation and until extraction for analysis.

2.8. Treatment of grain with enzyme ZHD518

Perforated and non-perforated contaminated wheat kernels were treated with enzyme or buffer, respectively, as outlined in Fig. 2. In short: 25 g of kernel was weighed into a 50 mL Falcon tube and wetted with either 5 mL 50 mM NaP pH 8.0 (control) or 5 mL 50mM NaP pH 8.0 containing 12 µg/mL purified ZHD518. Each sample (n=10) was mixed for 2 min immediately after adding the liquid to ensure equal distribution. The tubes were incubated for 24 h at 37 °C and the enzymatic reaction stopped by adding 5 mL methanol. The samples were stored at -20 °C until analysis.

2.9. Determination of ZEA contamination in grain samples

2.9.1. Samples and sampling procedure

A total of 120 samples (Fig. 2) were obtained and prepared for analysis. First, all the samples were freeze-dried for 48 hours (Martin Christ GmbH, Osterode am Harz, Germany). Then the wheat grains were ground in separate, closed, and disposable milling tubes using an IKA Tube-Mill 100 device (IKA-Werke GmbH, Staufen, Switzerland), under a fume hood. Each treatment condition, blanks and controls resulted in ten replicates. The ten replicates of blanks and control conditions were weighed out of pooled wheat flours, where the ten replicates of the different treatment conditions came from ten separated assays *(tubes)* (10 biological replicates) being extracted once (1 analytical replicate).

2.9.2. Extraction method

The samples were extracted according to Scarpino et al. (Scarpino et al., 2019) with some modifications: 5 g of wheat flour was weighed out in a 50 mL Falcon tube, and extracted with 20 mL of a mixture 79:20:1 (*v/v*) acetonitrile:water:acetic acid for 90 minutes in an overhead shaker at room temperature. The tubes were then centrifuged for 10 minutes at 4400 rpm. The supernatant was passed through a clean-up Oasis® Prime HLC cartridge (Waters AG, Baden, Switzerland) to remove fatty acids and phospholipids. No cartridge conditioning was performed.

First a 0.4 mL aliquot of the supernatant was passed through the column (3 cc, 150 mg) and discarded. A 1 mL aliquot of the supernatant was then passed through the column and collected. 5 μL of the cleaned extract was injected into the LC-MS/MS system.

2.9.3. HPLC-MS/MS analysis

LC-MS/MS analyses were conducted on a system consisting of a Thermo Vanquish Horizon chromatographic system coupled to an Altis Triple Quadrupole (TQ) mass spectrometer equipped with an electrospray ionization source (ESI) (Thermo Fisher Scientific, Reinach, Switzerland). Chromatographic separation was performed using an Agilent Poroshell 120 EC-C18 (2.1 × 100 mm, 2.7 μm) column protected by a guard column (Agilent EC-18, 2.1 × 5 mm, 2.7 μm) (Agilent Technologies, Basel, Switzerland).

The flow rate was set to 0.3 mL/min, and the column temperature set at 40 °C. The two elution mobile phases were made up of water + 0.1% (*v/v*) formic acid (mobile phase A) and methanol + 0.1% (*v/v*) formic acid (mobile phase B). Gradient elution was as follows: 0-0.5 min, 10% B; 6-15 min, 98% B; 15.10-23 min, 10% B. Injection volume was 5 μL.

The MS analyses were performed using Thermo Altis Triple Quadrupole instrument in negative ionisation mode (ESI -), in selected reaction monitoring (SRM) mode, alternating two transition reactions with the following settings: nitrogen served as the nebulising gas and argon as collision gas. The spray voltage was set at 3218 V, sheath gas was set at 42 (Arb), aux gas at 17 (Arb), sweep gas at 1 (Arb), ion transfer tube temperature at 350 °C; vaporizer temperature at 300 °C; CID gas at 1.5 mTorr.

MS tuning and optimisation of the MS/MS parameters for ZEA analysis were performed by means of direct infusion with a syringe of a separate standard solution of ZEA (5 mg/L) into the TQ using a syringe pump at a flow rate of 5 μL/min.

Optimized ESI-MS and ESI-MS/MS parameters and monitored transition reactions for ZEA are summarized in Table 1.

2.9.4. Matrix-matched calibration, LOD and LOQ

To quantify analytes accurately, a matrix-matched calibration was carried out by spiking 5 g of cleaned wheat grains (Zwicky, Müllheim-Wigoltingen, Switzerland; previously tested for ZEA under the detection limit as described in paragraph 2.9.3.) with 4 different concentrations of ZEA standard. The grains spiked with the mycotoxins were homogenized using a vortex (IKA Werke GmbH, Staufen, Switzerland) and allowed to rest overnight under the fume hood. On the next day the grains were ground and extracted using the same protocol as used for the samples, in triplicates.

ZEA: linear range: 100 μg/kg – 1 μg/kg.
Equation: $y = 12770x + 121.4$; $R^2 = 0.9974$.
LOD = 0.01 μg/kg (S/N = 3)
LOQ = 1 μg/kg (S/N = 10)

2.9.5. Accuracy and precision

To evaluate the accuracy and precision of the method, spiked samples in the blank matrix and matrix-matched samples (four spiking levels) were determined with 6 replicates. The average recovery was 91.4 % and the average relative standard deviation of the six replicates was 2.5 %. Both intraday and interday relative standard deviations were equal to 0.003 %.

Table 1
ESI-MS and ESI-MS/MS parameters and monitored transition reactions for the analysis of zearalenone.

Retention time (min)	Precursor ion (*m/z*)	Adduct ion	RF lens (V)	Products ions (*m/z*)	Collision energy (V)
6.18	317.38	$[M-H]^-$	84	174.97 (quan) 130.97 (qual)	23.8 29.5

2.9.6. Statistics

Statistical analysis was done using the XLSTAT statistical and data analysis solution for Excel (Premium Edition 2023.1.5). A one-way analysis of variance (ANOVA) followed by the Tukey's HSD test was carried out with the data from the treatment of grains with microorganisms and enzyme, and the level of significance was chosen as alpha = 0.05.

3. Results and Discussion

3.1. Preparation of grain samples and determination of wetting volume

In order to determine whether the core of the wheat kernels was made accessible to the enzyme or the microorganisms through the perforation, non-perforated and perforated kernels were soaked in excess liquid containing both the green-fluorescent AlexaFluor488 dye (mimicking the size of the enzyme ZHD518) and the red-fluorescent Nile Blue melamine resin beads (resembling the size of the microrganisms used) (Fig. 3A/B). Besides, perforated kernels were tempered with 5% liquid containing eGFP (a protein with a size comparable to ZHD518) for 24 h to evaluate the effect of reduced moisture conditions (Fig. 3C).

It is evident from Fig. 3 that neither of the two fluorescent particles pass the intact bran layers of the non-perforated kernels (image A), while both particles pass the bran layers after perforation (image B). Next, the optimal wetting volume was determined in accordance with typical wheat milling processing protocols in an industrial setting, where a small amount of liquid is added to wheat approximately 24 h before milling in order to optimise the separation of bran from endosperm. Diffusion of eGFP was determined at moisture concentrations of 2, 5, and 10%. The kernels showed different moisture contents after 24 h of wetting. The untreated sample had a moisture content of 11.7 % while the 2 %-sample, 5 %-sample and 10 %-sample had a content of 13.4 %, 15.3 % and 18.3 % respectively. The kernels also showed different degrees of diffusion of the eGFP solution. Kernels treated with 2 % solution showed no green fluorescence within the core, while clear fluorescence was seen in samples treated with 5 % and 10% solution. Fig. 3C shows that upon limited addition of liquid (5 %, containing only eGFP) the fluorescent particles pass the bran layers, but to a lesser extent than upon soaking in excess solution (Fig. 3B).

Kirsch & Odenthal (1999) suggested a total moisture content of 15-16 % to ensure effective downstream processing in milling. With an addition of 5 % wetting solution, a moisture content of 15.3 % was reached after 24 h and it could be shown through the use of eGFP, AlexaFluor488 and Nile Blue melamine resin bead that the enzyme and the microorganisms will presumably be able to diffuse into the core of perforated wheat kernels. Therefore 5 % of enzyme solution or microorganism suspension were used in the main trials.

3.2. Treatment with microorganisms

Wheat kernel were treated with solutions containing *Bacillus megaterium* Myk145 and *B. licheniformis* MA572, respectively, as outlined in Fig. 2. After 72 h of fermentation, the remaining ZEA contamination in the samples was quantified by mean of HPLC-MS/MS. The two strains were selected from a previous study (Mischler et al., 2024), where they showed high ZEA reduction (> 80 %) in a culture medium containing 0.1 μg/mL ZEA.

As can be observed in Fig. 4, neither *B. megaterium* Myk145 nor *B. licheniformis* MA572 significantly reduced the amount of ZEA in the perforated or non-perforated cereal grains compared to the controls after 72 hours of incubation at 30°C (see also Supplementary Material, Table S1). The cell counts revealed a starting concentration of 5 log CFU/g for *B. licheniformis* MA572 and 5.3 log CFU/g for *B. megaterium* Myk145. After 72 hours of incubation the cell count did not markedly

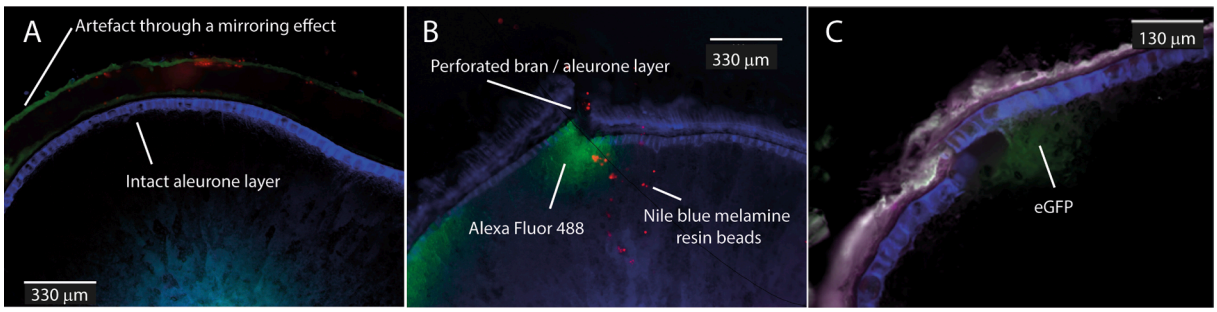

Fig. 3. *Fluorescence microscope images of wheat grains before perforation (A) and after 10 perforation cycles with a gap width of 0 mm (B) and (C). The kernels in (A) and (B) were soaked in 98 % ethanol containing green fluorescent AlexaFluor 488 (concentration 4.97 x 10^{14} beads per ml) for 4 h at 6°C, then dried at 25°C for 4 hours, then soaked in water containing red fluorescent Nile Blue melamine resin beads at a concentration of 2.23 x 10^{6} beads per ml for 4 h at 6°C. The kernels in (C) were wetted for 24 h with 5 % of green fluorescent eGFP and then stored for 24 h at 37 °C. Fluorescence microscopy was performed with an overlay of DAPI, FITC and TRITC filters.*

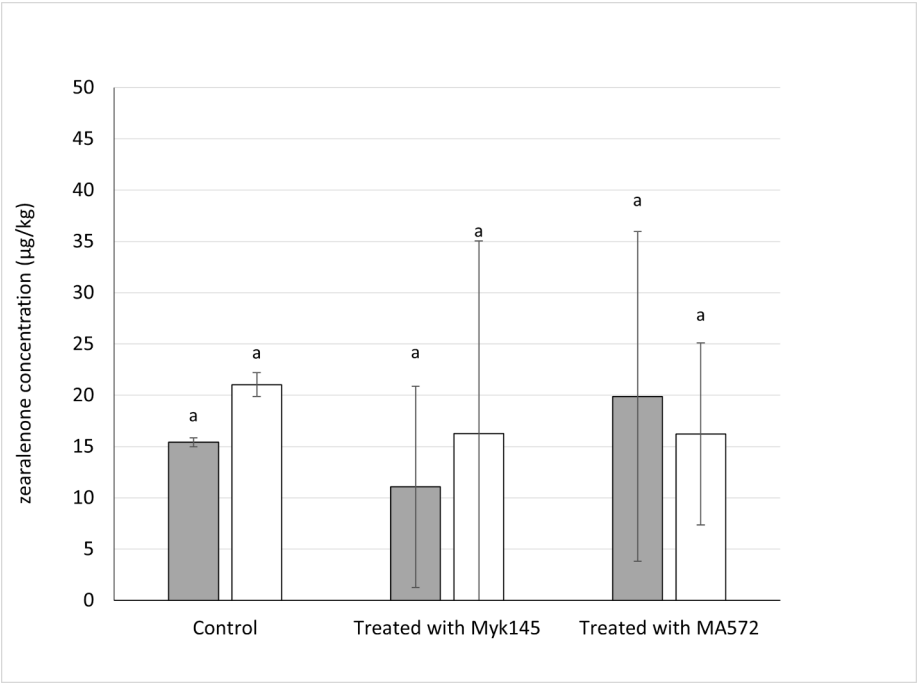

Fig. 4. Average concentration of zearalenone (µg/kg) in wheat kernels treated with Myk145 and MA572 microorganisms (n=10). Grey bars are standing for non-perforated grains; White bars are standing for perforated grains; a: ANOVA Tukey HSD with alpha = 0.05.

change, with 5.4 log CFU/g and 5.2 log CFU/g for MA572 and Myk145, respectively (Supplementary Material, Fig. S3). The MALDI-TOF analyses of the corresponding species from the wheat kernels fermented for 72 hours assumed that the original strains did not grow successfully, since *B. licheniformis* was only found in 50 % and *B. megaterium* in only 6 % of all isolates (Supplementary Material, Fig. S4). In a preliminary experiment to evaluate the optimal humidity for *B. licheniformis* MA572 and *B. megaterium* Myk145 to grow on wheat kernels, a 20% water addition (giving a 25% humidity and a aw value of 0.988) was sufficient for a cell growth of 2 log CFU/g after 24 h of fermentation (Supplementary Material, Fig. S2). Gauvry et al. also showed that this water activity value is sufficient for bacilli to grow (Gauvry et al., 2021). Therefore, a possible explanation for this unchanged cell count at 72 hours would be that the Bacillus cells may have died after 24 hours due to a possible loss of water during the longer fermentation time. On the other hand, these results may also indicate that the pore size created by cold needle perforation is not sufficient for bacteria to penetrate the grains, despite the positive results obtained in penetration tests with the fluorescent size marker depicted in Fig. 3. Moreover, these results may also shed light on the possible mechanism of action of these two microorganisms. The fact that a significant reduction in the concentration of zearalenone in the supernatant of a liquid culture medium was observed previously (Mischler et al., 2024) could indicate a binding mechanism of the mycotoxin by the two microorganisms and not a metabolizing action. This mitigation mechanism has been discussed previously (Sadiq et al., 2019). A detoxification mechanism involving a binding to the cell wall could be problematic, as a reversible binding could lead to detachment after processing and therefore consumption of the mycotoxin (Yiannikouris et al., 2004). A binding was often observed with lactic acid bacteria strains (Franco et al., 2011; Sadiq et al., 2019; Zou et al., 2012), whereas bacilli were described as able to do both adsorption and/or degradation (Cho et al., 2010; Jia et al., 2021). Nonetheless, degradation pathways of the toxin have to be assessed carefully to ensure that the degradation products are not toxic. ZEA has been shown for example to be metabolized to α- and β-zearalenol (ZOL), whereas α-ZOL would be more toxic than ZEA (Shier et al., 2001).

3.3. Treatment with enzyme

3.3.1. Enzyme activity and analysis of the degradation products in-vitro

Prior to application on the grains, the enzymatic activity of purified enzyme ZHD518 on zearalenone was tested *in-vitro* and the degradation products analyzed using HPLC-MS/MS.

Aliquots of the enzyme were checked for enzymatic activity towards ZEA at 40 °C in 50 mM NaP buffer pH 8.0 containing 25 µg/mL ZEA and 12 µg/mL purified enzyme (n=3). Reactions were run for 0.5 hours, 3 hours, 24 hours, and 48 hours. Remaining content of ZEA (Fig. 5A; as well as the content of the degradation products HZEA (Fig. 5B) and DHZEA (Fig. 5C) were determined by HPLC-MS/MS in samples with and without enzyme (control), respectively. Results for the degradation products are shown in relative peak area, as standard for neither HZEA nor DHZEA was commercially available for quantification. In the control sample (ZEA without enzyme) no degradation of ZEA was seen (Fig. 5A; white bars). In the presence of the enzyme ZHD518, ZEA concentrations dropped fast and after 24 hours of incubation, less than 0.1 % of the

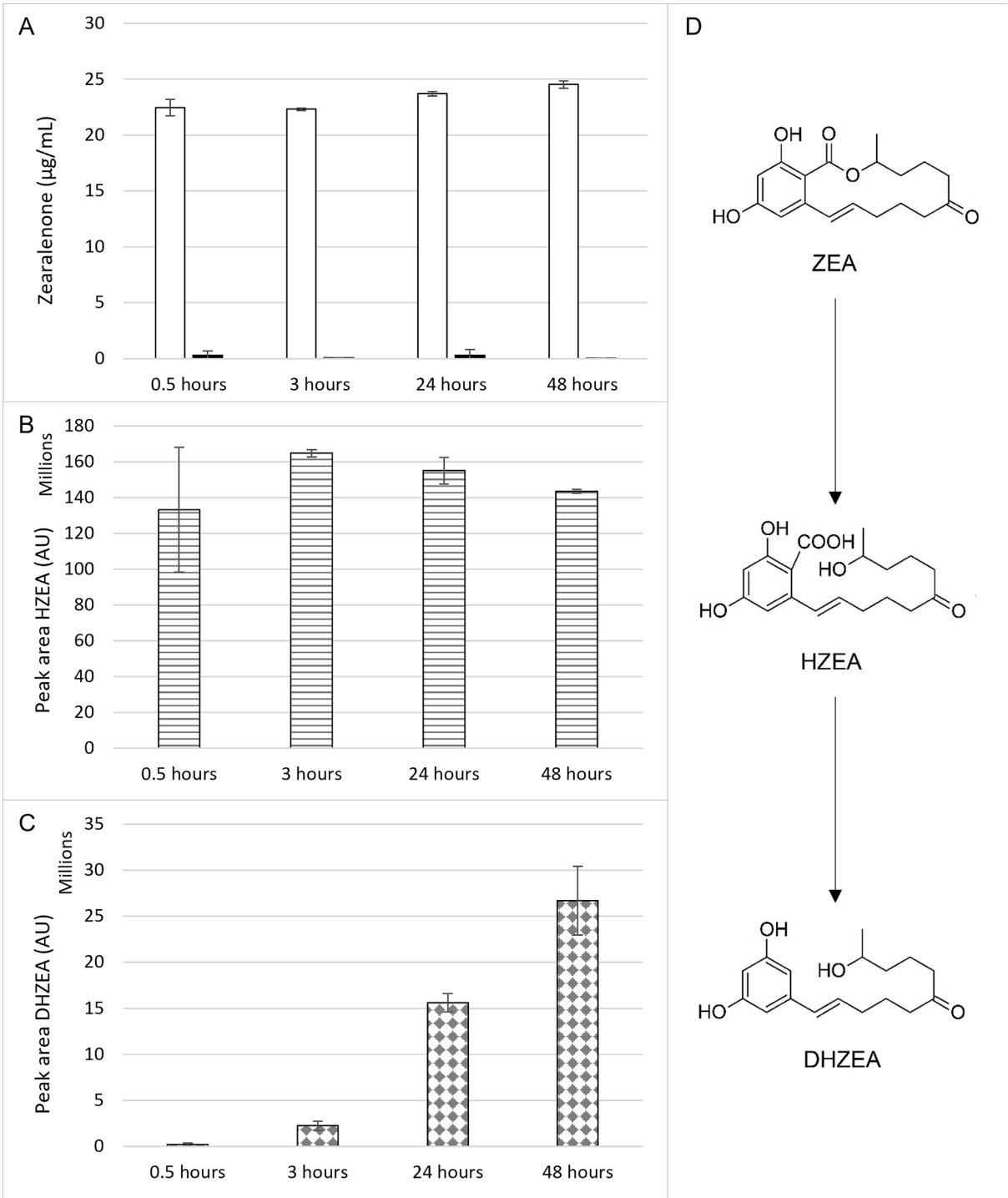

Fig. 5. (A) Concentration of remaining zearalenone (µg/mL) in control without enzyme (white bars) and with enzyme ZHD 518 (black bars) after in-vitro incubation for 0.5 hours, 3 hours, 24 hours, and 48 hours (n=3). (B) Peak area of chromatographic peak of the degradation products hydrolyzed zearalenone (HZEA) and (C) decarboxylated hydrolyzed zearalenone (DHZEA) after in vitro incubation with enzyme ZHD 518 for 0.5 hours, 3 hours, 24 hours and 48 hours (n=3). No metabolites were found in the control sample (data not shown). (D) Proposed pathway of zearalenone degradation by enzyme ZHD 518.

concentration of zearalenone had remained (Fig. 5A black bars). In treated, but not in untreated samples, two main metabolites (hydrolyzed zearalenone HZEA and decarboxylated hydrolyzed zearalenone DHZEA) were identified based on their exact masses and MS^2 fragmentation patterns in comparison with literature (Hahn et al., 2015; Krska et al., 2003; Vekiru et al., 2016) (Supplementary Material, Fig. S5 to S11). It is obvious that in the presence of the enzyme, ZEA is quickly transformed into HZEA (relative units, Panel B) and over time into DHZEA (relative units, Panel C).

Zearalenone hydrolase ZHD518 has 65% amino acid identity to the well-known ZEA-degrading enzyme ZHD101 (Wang et al., 2018). The degradation pathway of ZEA to HZEA via the cleavage of the lactone ring and the subsequent spontaneous decarboxylation of HZEA to DHZEA (Fig 5D) has been described earlier for ZHD101 (Kakeya et al., 2002; Vekiru et al., 2016) and could be assigned here to the enzyme ZHD518 for the first time *in-vitro* as well.

3.3.2. *Enzymatic treatment of infected grains*

Perforated and non-perforated infected grains were treated either with buffer or with an aqueous enzyme solution as outlined in Fig. 2. After 24 hours the remaining ZEA concentration in the samples was quantified using HPLC-MS/MS. In all enzyme-treated samples, the mycotoxin concentration was at least 80 % lower than in the buffer treated samples (controls) (Fig. 6). The effect of perforation on mitigation efficiency was not statistically significant.

High standard deviations were observed, due to the fact that in some samples the remaining ZEA concentration was very high (Table S1). This might be due to inhomogeneous distribution of the enzyme solution on the kernel surface in that particular sample. Another explanation could be an exceptionally high mycotoxin load in single grains, as high standard deviations have been seen in control samples without enzyme as well. Uneven distribution of infected kernels throughout a batch is a known problem. Mycotoxin contamination is typically accompanied by discoloring of the grain and/or deficiencies in grain development (Schaarschmidt & Fauhl-Hassek, 2018). Here grains were used as provided and the discolored or shrunken grains were not removed, which could explain the high standard deviations.

The distribution of zearalenone within our grain samples is not known, but it was reported that the content of mycotoxins within various parts of a single kernel varies (Schaarschmidt & Fauhl-Hassek, 2018). Since zearalenone is rather insoluble in water, the mycotoxin might accumulate within the bran fraction. Indeed, bran-containing by-products were shown to have higher mycotoxin concentration as products from the endosperm (Schaarschmidt & Fauhl-Hassek, 2018). Hence, the observed strong reduction of mycotoxin content after the perforation of the kernels (Fig. 6, controls, gray bar compared to white bar) might be caused by a separation of loose bran particles from the surface of the kernels during the cold needle perforation process.

Moreover, the enzymatic degradation was not complete. Within both perforated and non-perforated kernels, a concentration of about 5 µg/kg zearalenone remained. This could be addressed in the future by testing higher concentrations of enzyme or longer incubations times.

4. Conclusions

In order to reduce food waste, strategies are needed to mitigate mycotoxin contamination in grains in an efficient, time- and cost-effective way without compromising nutritional, sensorial or technological properties of wheat and products thereof. A biological mitigation strategy is presented in this study, where the combination of physical pre-treatment with cold needle perforation to facilitate, in a second step, access of biological decontamination agents (microorganisms or enzymes) to mycotoxins is suggested as a promising strategy to reduce food and feed waste. The enzyme approach applied in this study based on ZHD518 was successful. As enzyme reactions are highly specific, zearalenone degradation by ZHD518 results in two products that are no longer estrogen active, indicating that the material thus treated is no longer of concern. Further research will focus on optimizing treatment conditions regarding amount of enzyme used and incubation time. Since ZHD518 is a natural enzyme, optimizing its activity and stability by protein engineering is another focus of future research. In contrast, although the microorganisms-based approach is promising in solution, further investigations are currently being conducted such as to understand the mechanism of mycotoxin bio-detoxification of *B. megaterium*

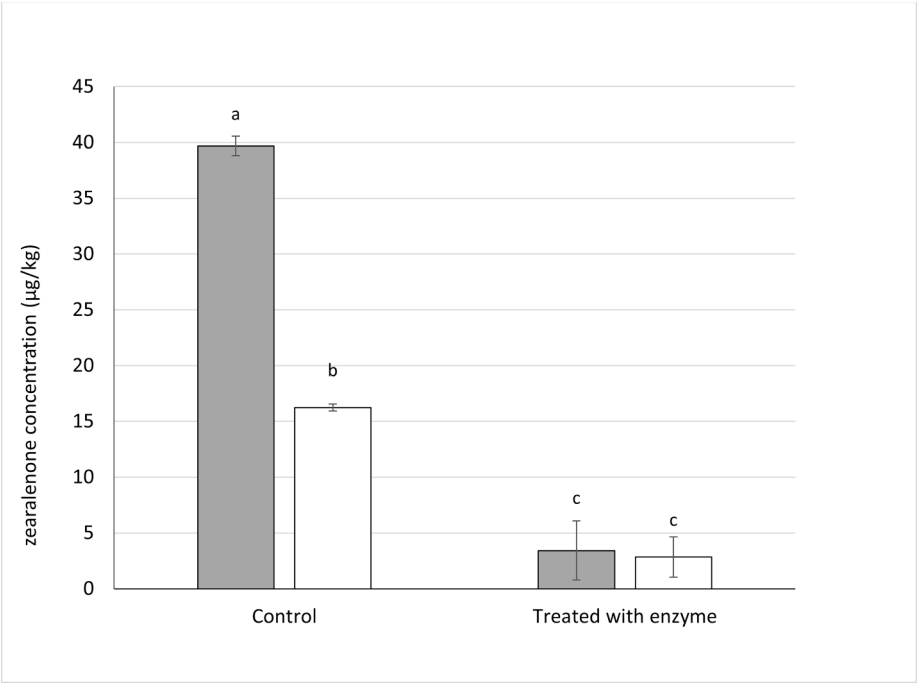

Fig. 6. Average concentration of zearalenone (µg/kg) in wheat kernels treated with enzyme ZHD518 (n=10). Grey bars represent non-perforated grains; White bars represent perforated grains; a,b.c: ANOVA Tukey HSD with alpha = 0.05.

Myk145 and *B. licheniformis* MA572 and will later support a targeted application.

While physical treatment does not seem to be necessary for the application of small enzymes, it is yet unclear whether the pores were not large enough for the microorganisms to pass or whether there was no sufficient water available to transport them into the kernel core.

A combination of physical and biological treatments could be a promising approach to address the global problem of mycotoxin contamination. Tests with raw materials other than wheat and contaminated with other mycotoxins are envisaged.

Funding

This work was supported by the ZHAW Health Research Hub.

CRediT authorship contribution statement

Amandine André: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Katrin Hecht:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Sandra Mischler:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Luca Stäheli:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Fllanza Kerhanaj:** Visualization, Validation, Investigation. **Rebecca Buller:** Supervision, Funding acquisition. **Mathias Kinner:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Susette Freimüller Leischtfeld:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Irene Chetschik:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Susanne Miescher Schwenninger:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Nadina Müller:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank all the collaborators from the research groups of food chemistry, food biotechnology, food technology and biocatalysis of the ZHAW for their support during the course of the research project. The authors would also like to thank John Bennett for proofreading this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114364.

References

André, A., Müller, N., & Chetschik, I. (2022). Occurrence of Zearalenone and Enniatin B in Swiss Wheat Grains and Wheat Flours. *Applied Sciences, 12*(20), Article 20. https://doi.org/10.3390/app122010566

Bennett, G. A., Lagoda, A. A., Shotwell, O. L., & Hesseltine, C. W. (1981). Utilization of zearalenone- contaminated corn for ethanol production. *Journal of the American Oil Chemists' Society, 58*(11), 974–976. https://doi.org/10.1007/BF02659774

Chang, X., Liu, H., Sun, J., Wang, J., Zhao, C., Zhang, W., Zhang, J., & Sun, C. (2020). Zearalenone Removal from Corn Oil by an Enzymatic Strategy. *Toxins, 12*(2), 117. https://doi.org/10.3390/toxins12020117

Chen, S.-W., Wang, H.-T., Shih, W.-Y., Ciou, Y.-A., Chang, Y.-Y., Ananda, L., Wang, S.-Y., & Hsu, J.-T. (2019). Application of Zearalenone (ZEN)-Detoxifying Bacillus in Animal Feed Decontamination through Fermentation. *Toxins, 11*(6), 330. https://doi.org/10.3390/toxins11060330

Cho, K. J., Kang, J. S., Cho, W. T., Lee, C. H., Ha, J. K., & Song, K. B. (2010). In vitro degradation of zearalenone by Bacillus subtilis. *Biotechnology Letters, 32*(12), 1921–1924. https://doi.org/10.1007/s10529-010-0373-y

Dänicke, S., & Winkler, J. (2015). Invited review: Diagnosis of zearalenone (ZEN) exposure of farm animals and transfer of its residues into edible tissues (carry over). *Food and Chemical Toxicology, 84*, 225–249. https://doi.org/10.1016/j.fct.2015.08.009

Döll, S., Dänicke, S., Ueberschär, K. H., Valenta, H., Schnurrbusch, U., Ganter, M., Klobasa, F., & Flachowsky, G. (2003). Effects of graded levels of Fusarium toxin contaminated maize in diets for female weaned piglets. *Archiv Fur Tierernahrung, 57*(5), 311–334. https://doi.org/10.1080/00039420310001607680

Efsa. (2011). Scientific Opinion on the risks for public health related to the presence of zearalenone in food. *Efsa Journal, 9*(6), 2197. https://doi.org/10.2903/j.efsa.2011.2197

EFSA Panel on Additives, Products or Substances used in Animal Feed (FEEDAP), Bampidis, V., Azimonti, G., Bastos, M. de L., Christensen, H., Dusemund, B., Fašmon Durjava, M., Kouba, M., López-Alonso, M., López Puente, S., Marcon, F., Mayo, B., Pechová, A., Petkova, M., Ramos, F., Sanz, Y., Villa, R. E., Woutersen, R., Bories, G., … López-Gálvez, G. (2022). Safety and efficacy of a feed additive consisting of zearalenone hydrolase produced by Escherichia coli DSM 32731 for all terrestrial animal species (Biomin GmbH). *EFSA Journal*, *20*(2). DOI: 10.2903/j.efsa.2022.7157.

Eskola, M., Kos, G., Elliott, C. T., Hajslova, J., Mayar, S., & Krska, R. (2019). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited "FAO estimate" of 25%. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2019.1658570

Commission, E. (2016). Appropriateness to set a group health-based guidance value for zearalenone and its modified forms. *EFSA Journal, 14*(4), e04425.

European Commission. (2023, April 25). Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. *Official Journal of the European Union*.

Fang, Y., Zhang, Z., Xu, W., Zhang, W., Guang, C., & Mu, W. (2022). Zearalenone lactonase: Characteristics, modification, and application. *Applied Microbiology and Biotechnology, 106*(21), 6877–6886. https://doi.org/10.1007/s00253-022-12205-5

FAO (Ed.). (2019). *Moving forward on food loss and waste reduction*.

Franco, T. S., Garcia, S., Hirooka, E. Y., Ono, Y. S., & Dos Santos, J. S. (2011). Lactic acid bacteria in the inhibition of Fusarium graminearum and deoxynivalenol detoxification: Bacterial removal of deoxynivalenol. *Journal of Applied Microbiology, 111*(3), 739–748. https://doi.org/10.1111/j.1365-2672.2011.05074.x

Fruhauf, S., Novak, B., Nagl, V., Hackl, M., Hartinger, D., Rainer, V., Labudová, S., Adam, G., Aleschko, M., Moll, W.-D., Thamhesl, M., & Grenier, B. (2019). Biotransformation of the Mycotoxin Zearalenone to its Metabolites Hydrolyzed Zearalenone (HZEN) and Decarboxylated Hydrolyzed Zearalenone (DHZEN) Diminishes its Estrogenicity In Vitro and In Vivo. *Toxins, 11*(8), 481. https://doi.org/10.3390/toxins11080481

Gauvry, E., Mathot, A.-G., Couvert, O., Leguérinel, I., & Coroller, L. (2021). Effects of temperature, pH and water activity on the growth and the sporulation abilities of Bacillus subtilis BSB1. *International Journal of Food Microbiology, 337*, Article 108915. https://doi.org/10.1016/j.ijfoodmicro.2020.108915

Gruber-Dorninger, C., Faas, J., Doupovec, B., Aleschko, M., Stoiber, C., Höbartner-Gußl, A., Schöndorfer, K., Killinger, M., Zebeli, Q., & Schatzmayr, D. (2021). Metabolism of Zearalenone in the Rumen of Dairy Cows with and without Application of a Zearalenone-Degrading Enzyme. *Toxins, 13*(2), 84. https://doi.org/10.3390/toxins13020084

Gruber-Dorninger, C., Jenkins, T., & Schatzmayr, G. (2019). Global Mycotoxin Occurrence in Feed: A Ten-Year Survey. *Toxins, 11*(7), 375. https://doi.org/10.3390/toxins11070375

Guan, Y., Lv, H., Wu, G., Chen, J., Wang, M., Zhang, M., Pang, H., Duan, Y., Wang, L., & Tan, Z. (2023). Effects of Lactic Acid Bacteria Reducing the Content of Harmful Fungi and Mycotoxins on the Quality of Mixed Fermented Feed. *Toxins, 15*(3), Article 3. https://doi.org/10.3390/toxins15030226

Hahn, I., Kunz-Vekiru, E., Twaruzek, M., Grajewski, J., Krska, R., & Berthiller, F. (2015). Aerobic and anaerobic in vitro testing of feed additives claiming to detoxify deoxynivalenol and zearalenone. *Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment, 32*(6), 922–933. https://doi.org/10.1080/19440049.2015.1023741

Han, X., Huangfu, B., Xu, T., Xu, W., Asakiya, C., Huang, K., & He, X. (2022). Research Progress of Safety of Zearalenone: A Review. *Toxins, 14*(6), 386. https://doi.org/10.3390/toxins14060386

Hassan, Z. U., Al Thani, R., Alsafran, M., Migheli, Q., & Jaoua, S. (2021). Selection of Bacillus spp. With decontamination potential on multiple Fusarium mycotoxins. *Food Control, 127*, Article 108119. https://doi.org/10.1016/j.foodcont.2021.108119

Ji, J., Yu, J., Xu, W., Zheng, Y., Zhang, Y. Z., & Sun, X. L. (2022). Isolation and Mechanistic Characterization of a Novel Zearalenone-Degrading Enzyme. *Foods, 11* (18). https://doi.org/10.3390/foods11182908

Jia, R., Cao, L. R., Liu, W. B., & Shen, Z. Y. (2021). Detoxification of deoxynivalenol byBacillus subtilisASAG 216 and characterization the degradation process. *European Food Research and Technology, 247*(1), 67–76. https://doi.org/10.1007/s00217-020-03607-8

Kakeya, H., Takahashi-Ando, N., Kimura, M., Onose, R., Yamaguchi, I., & Osada, H. (2002). Biotransformation of the Mycotoxin, Zearalenone, to a Non-estrogenic Compound by a Fungal Strain of *Clonostachys* sp. *Bioscience, Biotechnology, and Biochemistry, 66*(12), 2723–2726. https://doi.org/10.1271/bbb.66.2723

Kirsch, B., & Odenthal, A. (1999). Fachkunde Müllereitechnologie - Werkstoffkunde: Ein Lehrbuch über die Zusammensetzung, Untersuchung, Bewertung und Verwendung von Getreide und Getreideprodukten (4., erw. *Aufl). Bayerischer Müllerbund.*

Krska, R., Welzig, E., Josephs, R. D., Kandler, W., Pettersson, H., MacDonald, S., Charlton, A., Brereton, P., Hametner, C., Berner, D., & Zöllner, P. (2003). Purity Assessment of Crystalline Zearalenone. *Journal of AOAC INTERNATIONAL, 86*(4), 722–728. https://doi.org/10.1093/jaoac/86.4.722

Laca, A., Mousia, Z., Diaz, M., Webb, C., & Pandiella, S. S. (2006). Distribution of microbial contamination within cereal grains. *Journal of Food Engineering, 72*(4), 332–338. https://doi.org/10.1016/j.jfoodeng.2004.12.012

Liu, M., Zhao, L., Gong, G., Zhang, L., Shi, L., Dai, J., Han, Y., Wu, Y., Khalil, M. M., & Sun, L. (2022). Invited review: Remediation strategies for mycotoxin control in feed. *Journal of Animal Science and Biotechnology, 13*(1), 19. https://doi.org/10.1186/s40104-021-00661-4

Liu, X., Wu, N., Zhang, M., Xue, F., & Xu, Q. (2023). Isolation and Characterization of the Zearalenone-Degrading Strain, Bacillus spizizenii B73, Inspired by Esterase Activity. *Toxins, 15*(8), Article 8. https://doi.org/10.3390/toxins15080488

Luo, S. J., Du, H. L., Kebede, H., Liu, Y., & Xing, F. G. (2021). Contamination status of major mycotoxins in agricultural product and food stuff in Europe. *Food Control, 127*, 14. https://doi.org/10.1016/j.foodcont.2021.108120

Makkar, H. P. S. (2014). Biofuel co-products as livestock feed. Opportunities and challenges, technical summary. *FAO*.

Minervini, F., Giannoccaro, A., Cavallini, A., & Visconti, A. (2005). Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. *Toxicology Letters, 159*(3), 272–283. https://doi.org/10.1016/j.toxlet.2005.05.017

Mischler, S., André, A., Freimüller Leischtfeld, S., Müller, N., Chetschik, I., & Miescher Schwenninger, S. (2024). Potential of Lactic Acid Bacteria and Bacillus spp. In a Bio-Detoxification Strategy for Mycotoxin Contaminated Wheat Grains. *Applied Microbiology, 4*(1), Article 1. https://doi.org/10.3390/applmicrobiol4010007

Molina-Molina, J.-M., Real, M., Jimenez-Diaz, I., Belhassen, H., Hedhili, A., Torné, P., Fernández, M. F., & Olea, N. (2014). Assessment of estrogenic and anti-androgenic activities of the mycotoxin zearalenone and its metabolites using in vitro receptor-specific bioassays. *Food and Chemical Toxicology, 74*, 233–239. https://doi.org/10.1016/j.fct.2014.10.008

Muhialdin, B. J., Saari, N., & Meor Hussin, A. S. (2020). Review on the Biological Detoxification of Mycotoxins Using Lactic Acid Bacteria to Enhance the Sustainability of Foods Supply. *Molecules, 25*(11), 2655. https://doi.org/10.3390/molecules25112655

Olsen, M., Mirocha, C. J., Abbas, H. K., & Johansson, B. (1986). Metabolism of High Concentrations of Dietary Zearalenone by Young Male Turkey Poults. *Poultry Science, 65*(10), 1905–1910. https://doi.org/10.3382/ps.0651905

Orlando, B., Grignon, G., Vitry, C., Kashefifard, K., & Valade, R. (2019). Fusarium species and enniatin mycotoxins in wheat, durum wheat, triticale and barley harvested in France. *Mycotoxin Research, 35*(4), 369–380. https://doi.org/10.1007/s12550-019-00363-x

Pierzgalski, A., Bryła, M., Kanabus, J., Modrzewska, M., & Podolska, G. (2021). Updated Review of the Toxicity of Selected Fusarium Toxins and Their Modified Forms. *Toxins, 13*(11), 768. https://doi.org/10.3390/toxins13110768

Rogowska, A., Pomastowski, P., Sagandykova, G., & Buszewski, B. (2019). Zearalenone and its metabolites: Effect on human health, metabolism and neutralisation methods. *Toxicon, 162*, 46–56. https://doi.org/10.1016/j.toxicon.2019.03.004

Sadiq, F. A., Yan, B., Tian, F., Zhao, J., Zhang, H., & Chen, W. (2019). Lactic Acid Bacteria as Antifungal and Anti-Mycotoxigenic Agents: A Comprehensive Review. *Comprehensive Reviews in Food Science and Food Safety, 18*(5), 1403–1436. https://doi.org/10.1111/1541-4337.12481

Scarpino, V., Reyneri, A., & Blandino, M. (2019). Development and Comparison of Two Multiresidue Methods for the Determination of 17 Aspergillus and Fusarium Mycotoxins in Cereals Using HPLC-ESI-TQ-MS/MS. *Frontiers in Microbiology, 10*, 361. https://doi.org/10.3389/fmicb.2019.00361

Schaarschmidt, S., & Fauhl-Hassek, C. (2018). The Fate of Mycotoxins During the Processing of Wheat for Human Consumption. *Comprehensive Reviews in Food Science and Food Safety, 17*(3), 556–593. https://doi.org/10.1111/1541-4337.12338

Shcherbakova, L., Rozhkova, A., Osipov, D., Zorov, I., Mikityuk, O., Statsyuk, N., Sinitsyna, O., Dzhavakhiya, V., & Sinitsyn, A. (2020). Effective Zearalenone Degradation in Model Solutions and Infected Wheat Grain Using a Novel Heterologous Lactonohydrolase Secreted by Recombinant Penicillium canescens. *Toxins, 12*(8), 475. https://doi.org/10.3390/toxins12080475

Shier, W. T., Shier, A. C., Xie, W., & Mirocha, C. J. (2001). Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicon, 39* (9), 1435–1438. https://doi.org/10.1016/S0041-0101(00)00259-2

Średnicka, P., Juszczuk-Kubiak, E., Wójcicki, M., Akimowicz, M., & Roszko, M.Ł. (2021). Probiotics as a biological detoxification tool of food chemical contamination: A review. *Food and Chemical Toxicology, 153*, Article 112306. https://doi.org/10.1016/j.fct.2021.112306

Stäheli, L., Schwab, L., Kinner, M., & Müller, N. (2023). Design and Functionality of a Prototype for Cold Needle Perforation of Wheat. *Applied Sciences, 13*(10), 6266. https://doi.org/10.3390/app13106266

Stanciu, O., Juan, C., Miere, D., Loghin, F., & Mañes, J. (2017). Presence of Enniatins and Beauvericin in Romanian Wheat Samples: From Raw Material to Products for Direct Human Consumption. *Toxins, 9*(6), 189.

Studier, F. W. (2005). Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification, 41*(1), 207–234. https://doi.org/10.1016/j.pep.2005.01.016

Takahashi-Ando, N., Kimura, M., Kakeya, H., Osada, H., & Yamaguchi, I. (2002). A novel lactonohydrolase responsible for the detoxification of zearalenone: Enzyme purification and gene cloning. *Biochemical Journal, 365*(1), 1–6. https://doi.org/10.1042/bj20020450

Takahashi-Ando, N., Ohsato, S., Shibata, T., Hamamoto, H., Yamaguchi, I., & Kimura, M. (2004). Metabolism of Zearalenone by Genetically Modified Organisms Expressing the Detoxification Gene from *Clonostachys rosea*. *Applied and Environmental Microbiology, 70*(6), 3239–3245. https://doi.org/10.1128/AEM.70.6.3239-3245.2004

Vekiru, E., Fruhauf, S., Hametner, C., Schatzmayr, G., Krska, R., Moll, W. D., & Schuhmacher, R. (2016). Isolation and characterisation of enzymatic zearalenone hydrolysis reaction products. *World Mycotoxin Journal, 9*(3), 353–363. https://doi.org/10.3920/WMJ2015.2005

Vogelgsang, S., Musa, T., Bänziger, I., Kägi, A., Bucheli, T. D., Wettstein, F. E., Pasquali, M., & Forrer, H.-R. (2017). Fusarium Mycotoxins in Swiss Wheat: A Survey of Growers' Samples between 2007 and 2014 Shows Strong Year and Minor Geographic Effects. *Toxins*, *9*(8), Article 8. DOI: 10.3390/toxins9080246.

Wang, M., Yin, L., Hu, H., Selvaraj, J. N., Zhou, Y., & Zhang, G. (2018). Expression, functional analysis and mutation of a novel neutral zearalenone-degrading enzyme. *International Journal of Biological Macromolecules, 118*, 1284–1292. https://doi.org/10.1016/j.ijbiomac.2018.06.111

Wu, F., & Munkvold, G. P. (2008). Mycotoxins in Ethanol Co-products: Modeling Economic Impacts on the Livestock Industry and Management Strategies. *Journal of Agricultural and Food Chemistry, 56*(11), 3900–3911. https://doi.org/10.1021/jf072697e

Yiannikouris, A., François, J., Poughon, L., Dussap, C.-G., Bertin, G., Jeminet, G., & Jouany, J.-P. (2004). Adsorption of Zearalenone by β-d-Glucans in the Saccharomyces cerevisiae Cell Wall. *Journal of Food Protection, 67*(6), 1195–1200. https://doi.org/10.4315/0362-028X-67.6.1195

Zheng, Y., Liu, W., Chen, C.-C., Hu, X., Liu, W., Ko, T.-P., Tang, X., Wei, H., Huang, J.-W., & Guo, R.-T. (2018). Crystal Structure of a Mycoestrogen-Detoxifying Lactonase from *Rhinocladiella mackenziei*: Molecular Insight into ZHD Substrate Selectivity. *ACS Catalysis, 8*(5), 4294–4298. https://doi.org/10.1021/acscatal.8b00464

Zou, Z.-Y., He, Z.-F., Li, H.-J., Han, P.-F., Meng, X., Zhang, Y., Zhou, F., Ouyang, K.-P., Chen, X.-Y., & Tang, J. (2012). In vitro removal of deoxynivalenol and T-2 toxin by lactic acid bacteria. *Food Science and Biotechnology, 21*(6), 1677–1683. https://doi.org/10.1007/s10068-012-0223-x