TITLE: Challenges of *Lactobacillus* fermentation in combination with acoustic screening for deoxynivalenol and deoxynivalenol conjugates reduction in contaminated wheat-based products

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Challenges of Lactobacillus Fermentation for Deoxynivalenol and Deoxynivalenol Conjugates Elimination from Contaminated Wheat Grains

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Abstract:	This study was dedicated to apply biological treatment using Lactobacillus (LAB) fermentation separately or in combination with an acoustic screening method for the prevention of mycotoxins in Fusarium spp. contaminated wheat grains. Wheat grain samples of different contamination were treated separately using antimicrobial LAB strains (L. casei, L. plantarum, L. paracasei, and L. uvarum) and the changes on the level of deoxynivalenol (DON) and its conjugates such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and DON-3--D-glucoside (D3G)) were evaluated using UHPLC-QqQ-MS/MS and UHPLC-Orbitrap-HRMS. Additionally, an acoustic device was used to analyse DON in the wheat raw samples (without treatment). High linear correlations were obtained between HPLC results and the penetrated acoustic signal amplitude (Ap) for DON and D3G (R2 = 0.85 and R2 = 0.82, respectively). The results of fermentation demonstrated that bio-treatment of contaminated wheat was very effective for DON and masked toxin reduction/or elimination from the media. Contaminated wheat grain fermentation using L. uvarum allowed to reduce DON and D3G content in the media up to 75.0% and 84.1%, respectively, while DON conjugates (3-ADON, 15-ADON) were completely eliminated. Fusarium spp. contaminated wheat grains demonstrated different enzymatic profiles (amylolytic, xylanolytic, and proteolytic) which could be related with biological degradation of mycotoxins during fermentation. The amylolytic and xylanolytic activities of fungi correlated well with DON content (R2 = 0.8235, R2 = 0.8694, respectively) as well as with D3G (R2 = 0.9314, R2 = 0.9937, respectively). The findings of this study indicate that bio-treatment of contaminated wheat could efficiently reduce Fusarium mycotoxin levels in wheat grain and improve the sustainability of grain production. The acoustic technique could identify DON as well as D3G contamination in raw wheat grains and is a promising tool in the wheat grain processing chain.
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Highlights:

- LAB fermentation decreased the amount of D3G in the media, on average, by 84.1%.
- *In situ Lactobacillus* fermentation of wheat reduced Deoxynivalenol by 37%.
- The most effective biological detoxification can be reached using *L. uvarum*.
- Rapid acoustic screening can be applied for DON and D3G analysis.

Challenges of Lactobacillus Fermentation for Deoxynivalenol and Deoxynivalenol Conjugates Elimination from Contaminated Wheat Grains

Running Title: *Fusarium* spp. Contaminated Wheat Grains Detoxification using *Lactobacillus* Fermentation

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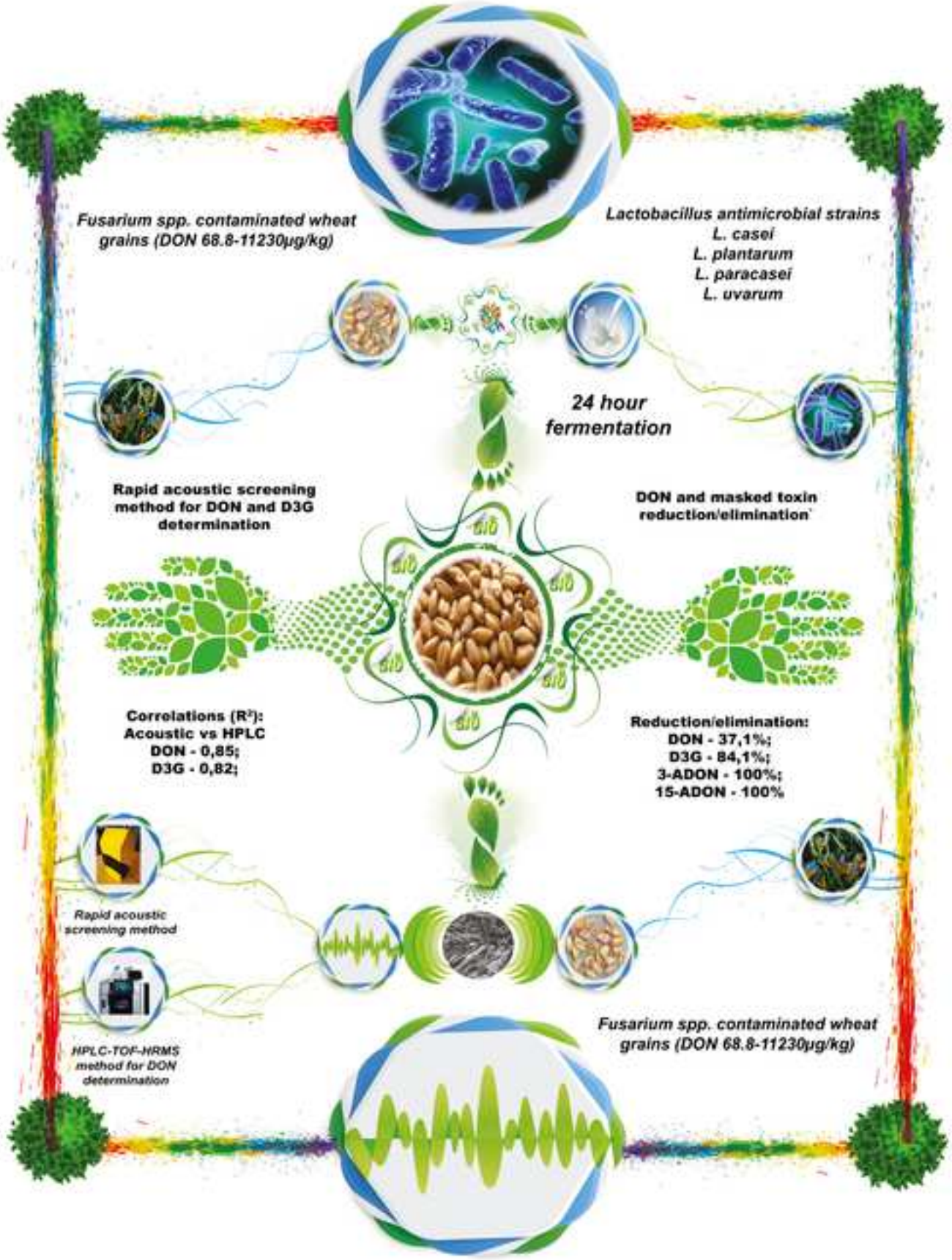
Graphical abstract



ABSTRACT

This study was dedicated to apply biological treatment using *Lactobacillus* (LAB) fermentation separately or in combination with an acoustic screening method for the prevention of mycotoxins in *Fusarium* spp. contaminated wheat grains. Wheat grain samples of different contamination were treated separately using antimicrobial LAB strains (*L. casei*, *L. plantarum*, *L. paracasei*, and *L. uvarum*) and the changes on the level of deoxynivalenol (DON) and its conjugates such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and DON-3-β-D-glucoside (D3G)) were evaluated using UHPLC-QqQ-MS/MS and UHPLC-Orbitrap-HRMS. Additionally, an acoustic device was used to analyse DON in the wheat raw samples (without treatment). High linear correlations were obtained between HPLC results and the penetrated acoustic signal amplitude (Ap) for DON and D3G (R^2 = 0.85 and R^2 = 0.82, respectively). The results of fermentation demonstrated that bio-treatment of contaminated wheat was very effective for DON and masked toxin reduction/or elimination from the media. Contaminated wheat grain fermentation using *L. uvarum* allowed to reduce DON and D3G content in the media up to 75.0% and 84.1%, respectively, while DON conjugates (3-ADON, 15-ADON) were completely eliminated. *Fusarium* spp. contaminated wheat grains demonstrated different enzymatic profiles (amylolytic, xylanolytic, and proteolytic) which could be related with biological degradation of mycotoxins during fermentation. The amylolytic and xylanolytic activities of fungi correlated well with DON content (R^2 = 0.8235, R^2 = 0.8694, respectively) as well as with D3G (R^2 = 0.9314, R^2 = 0.9937, respectively). The findings of this study indicate that bio-treatment of contaminated wheat could efficiently reduce *Fusarium* mycotoxin levels in wheat grain and improve the sustainability of grain production. The acoustic technique could identify DON as well as D3G contamination in raw wheat grains and is a promising tool in the wheat grain processing chain.

Keywords: *Fusarium* spp.; biological detoxification; deoxynivalenol and masked toxins; acoustic sensors; antimicrobial properties of *Lactobacillus*;

1. Introduction

The contamination of crops with mycotoxins is worldwide problem which impacts human and animal health and causes economical losses due to the relevant damage for the food and feed processing chain (Stanciu et el., 2015; Guo et al., 2020). DON has drawn global attention as it is one of the widest spread toxins of cereals and cereal-based products in food/feed (Alizadeh et al., 2016; Gruber-Dorninger et al., 2019). Contamination with DON is different in various continents and according to results from the 2019 mycotoxin survey in Europe more than half of wheat samples (54.5%) were contaminated with DON (Biomin, 2019). Consequently, strategies to reduce or eliminate DON in food/feed needs to be developed.

Deoxynivalenol (DON) is a secondary metabolite product of *Fusarium* spp. and belongs to type B trichothecenes class. *Fusarium spp*. produces not only DON, but also two acetylated DON metabolites such as 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON). Apart from these fungi metabolites, masked mycotoxins, with unpredictable toxicity, can be formed by a plant as response to a fungal infection obtaining plant-derived DON-glucoside (D3G). Masked mycotoxins appears to be dominant co-contaminants in cereals, especially in wheat and barley, as well as in rye grains (Maul et al., 2012). DON is highly susceptible to transformation, and the mutual transformation and formation of these masked mycotoxins, particularly of D3G, can be potentially risky (Gratz 2017; Michlmayr et al., 2017; Freire & Sant'Ana, 2018).

There is a lack of information about the toxicity of DON conjugates and their impact on human or animal health. As it is reported by Juan-Garcia et. al. (2018, 2019) 15-ADON is less toxic than 3-ADON, however, other studies found contrary results and indicated 15-ADON to

be more toxic (Pinton et al., 2012). According to European Food Safety Authority (EFSA, 2014) acetylated derivatives of DON (3-ADON and 15-ADON) might cause the same acute and chronic effects as parent toxin DON. Furthermore, DON-3-glucoside can be reversed back to DON by bacteria in the human gastrointestinal tract and reactivate its toxicity (EFSA, 2019).

In humans as well as in animals, DON reduces food intake and nutrient absorption, induces vomiting. Lower intake of food is caused by the intestinal factors, including the production of pro-inflammatory cytokines and hormones (Terciolo et al., 2018). Concerning the protection of consumers from health risks related to the intake of mycotoxins, national and European legislative (European Commission, 2006) institutions have instated maximum tolerable levels of DON in unprocessed cereals (1750 µg/kg), cereals intended for direct human consumption and cereal flour (750 µg/kg), pasta (750 µg/kg), bread and other baked goods (500 µg/kg), processed cereal-based foods (200 µg/kg). Due to high portions of D3G, transformed to its free form during digestion, masked toxins should be added to total DON exposure. In Europe, D3G is routinely monitored, and European Food Safety Authority (EFSA) concludes a maximum tolerable daily intake (TDI) of 1 µg/kg body weight/day for the sum of DON and its conjugates (3-ADON, 15-ADON, and D3G) (EFSA, 2019).

However, it can be difficult to control DON contamination (AWAD et al., 2010) because its accumulation in grain is significantly affected by environmental factors, occurring in the field and spreading from pre-harvest to processing (Pitt et al., 2013). Therefore, the detection of DON and its modified forms and prevention tools via inhibition of mycotoxin formation in the earliest stage of the cereal processing chain should be seriously considered (Berthiller et al., 2013; Brodehl et al., 2014). According to the results of Information Technology for European Advancement 2 (ITEA2) Eureka project ACOUSTICS, considerable success in controlling DON in wheat grains could be achieved using a rapid acoustic method and developed acoustic technique (Juodeikiene et. al., 2014).

Assessing the major hazard of *Fusarium* mycotoxins to food safety and human/animal health, new strategies for biological detoxification of crops, such as wheat, is relevant. Approaches used to reduce mycotoxins produced by *Fusarium* high blind can be classified into three categories: physical, chemical, and biological. However, the first two categories have limited practical possibilities (Peng et al., 2018). Recently, the application of biodegradation methods in food and feed processing has been a significant area of research. Bio-preservation of grains, using microorganisms or their metabolites (acids, enzymes) resulted in inhibited synthesis, absorbtion and structural destruction of DON, as well as enhanced safety of feed by extending the shelf life (Pfliegler et al., 2015; Gao et al., 2018).

Fermentation is one of the oldest food processing technologies which is widely applied in food/feed preparation and positively influences nutritional, sensory and textural properties as well as increases the shelf life of final products (Verni et al., 2020). Thus, the application of lactic acid bacteria and their metabolites is a promising biotechnology to control mould development and mycotoxin accumulation in contaminated grains and by-products.

The mechanism of mycotoxin biological degradation can be related with microbial conjugation as well as with enzymatic degradation (Ji et al., 2016; Li et al., 2020). This study hypothesizes whether the decrease in mycotoxin concentrations could be related with the enzymatic system of fungi in *Fusarium* spp. contaminated wheat at the initial stage which could also affect the fermentation process as well as with the application of LAB with absorption capabilities. Therefore, it is necessary to select microorganisms with the highest ability to multiply in contaminated media in order to achieve the most effective biological detoxification. Despite the growing interest to masked mycotoxins, their literature analysis, express screening, and changes during fermentation, there is still lack of essential information to establish effective prevention and control actions. Thus so far, acoustic sensors have been tested for the detection of DON masked conjugates.

The main goals of this experiment are: (i) to study the effect of biological treatment using LAB strains with antimicrobial activity on the changes of DON and its conjugates in *Fusarium* spp. contaminated wheat grain; (ii) to evaluate the enzymatic activities of contaminated wheat grains and their relationships with mycotoxin concentrations; (iii) to apply the acoustic method for DON and its conjugates detection in contaminated cereals.

2. Materials and Methods

2.1 Samples

Uncontaminated wheat samples, as well as those infected by *Fusarium* spp., with different levels of DON were were collected in the 2019 wheat harvest season from Northern Serbia, the Autonomous Province of Vojvodina. The selection of wheat samples for this study was based on naturally contaminated wheat samples with DON to covered a wide range of its concentrations. The highest amount of cereals (wheat, maize, barley), in the Republic of Serbia, is produced in Northern Serbia, which classifies this region as the main cereals growing area. The level of contamination was determined by high-performance liquid chromatography (HPLC-TOF-HRMS, Bruker, Germany) and is presented in Table 1. All samples were placed in a sealed plastic bags, transferred in a cooler (4-6°C) and stored in a freezer at -18°C.

2.2 High-Performance Liquid Chromatography

High-performance liquid chromatography coupled to time of flight high-resolution mass spectrometry (HPLC-TOF-HRMS, Bruker, Germany) was applied for mycotoxin (in μg/kg) analysis. The following mycotoxins, in wheat grain samples, were analysed: DON, D3G, 3 - ADON and 15 - ADON.

The samples were prepared using a modified QuEChERS method. HPLC-TOF-HRMS analysis was performed on an UltiMate 3000 (Thermo Fisher Scientific, USA) High performance liquid chromatography (HPLC) system coupled to a compact Q-ToF time-of-flight mass spectrometer (Bruker, Germany). Chromatographic separation was performed on a reversed-phase analytical column (Kinetex C_{18}, 1.7 µm, 100 Å, 50 × 3.00 mm; Phenomenex, USA) at a 0.35 mL min^{-1} flow rate. The analysis was performed in positive full scan mode for all mycotoxins over the *m/z* scanning range from 50 to 1000. The mass extraction window applied for quantification purposes was set to ± 5 ppm at 10,000 full-width half-maximum (FWHM) resolution. Data acquisition was controlled by HyStar 3.2 software (Bruker Daltonik GmbH, Bremen, Germany), and data analysis was performed with QuantAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany). Mycotoxin detection limits were as follows: DON (*m*-LOD 20 µg/kg), D3G (*m*-LOD 20 µg/kg), 3-ADON (*m*-LOD 10 µg/kg), and 15-ADON (*m*-LOD 10 µg/kg).

2.3 Acoustic Method for DON Screening in Cereals

DON contamination levels in wheat samples (12–13% humidity, determined using AACC method 44-15, 2000) were screened using a brand new at-line and off-line portable acoustic spectrometer with penetration (Juodeikiene et. al., 2014). The spectrometer measures, in relative units, the amplitude of the acoustic signal (Ap) that penetrates the sample matrix over the frequency range of 10–60 kHz. The duration of each measurement was ~10 seconds. The test was carried out by placing a 200 g portion of sample grains into a plastic vessel, whose base was covered by sound-transmitting material. The thickness of the grain layer was 50 mm, with a diameter of 80 mm. The impact of DON, determined by HPLC, and Ap was performed.

2.4 Enzymatic Profiles of Wheat Grain Samples

150 *Amylolytic* activity was determined according to ICC Standard Method 108 (ICC, 1998).
151 *Xylanolytic activity* was determined using the DNS (3.5-dinitrosalicylic acid) method (Bailey
152 et al., 1992). One unit of endoxylanase activity (XU) was defined as the amount of enzyme
153 required to release 1 micromole of xylose equivalents from 1% birchwood endoxylan (Roth,
154 Germany) per 1 minute under the assay conditions used (pH 4.5, 40ºC). *Proteolytic activity*
155 was carried out as described by (Cupp-Enyard, 2008) using 0.65% (w/v) casein as a substrate.
156
157 *2.5 LAB Fermentation Process of Wheat Grain Samples*
158 Five different LAB strains (*Lactobacillus brevis* No. 173, *Lactobacillus casei* No. 210,
159 *Lactobacillus plantarum* No. 135, *Lactobacillus paracasei* No. 244, and *Lactobacillus uvarum*
160 No. 245), isolated from a spontaneous rye sourdough, were provided by the Lithuanian
161 University of Health Sciences (Bartkiene et al., 2020). The LAB samples were stored at -70ºC
162 in 25% glycerol solution. LAB samples were refreshed and propagated in a de Man, Rogosa,
163 and Sharpe (MRS) broth (CM 0359, Oxoid Ltd, Hampshire, UK) for 48 hours at their optimal
164 temperature (30°C).
165 Wheat samples (W1 - W6) were grounded in a laboratory mill (Bühler-Miag Brunswick,
166 Germany) and used for LAB fermentation. Each sample (50 g) was mixed with distilled water
167 to reach moisture of 65%, then a 3% (w/v) of the LAB cells were inoculated into a fresh
168 medium. Inoculated wheat samples were incubated anaerobically under stationary conditions
169 for 24h at 30°C in the thermostat (TC160, SalvisLab Thermocenter, IL, USA). During LAB
170 fermentation, the grain samples were subjected to microbiological and enzymatic profiles and
171 pH analysis. The determinations of LAB and mould/yeast (M/Y) counts in by-products were
172 performed according to (Bartkiene et al., 2019). The residue of the fermented wheat samples
173 was lyophilized following fermentation and stored under dry, dark conditions for mycotoxin

analysis by HPLC-TOF-HRMS. Initial contaminated cereal samples without fermentation were used as a control.

For acidity analysis of fermentation process a pH electrode was used for pH measurements (PP-15; Sartorius, Göttingen, Germany).

2.6 Statistical Analysis

The results were expressed as the mean (n = 5) ± standard deviation (SD). To evaluate the effects of the different by-products, their different treatments, and the quantity of additives used on bread quality parameters, the data were analysed by multivariate analysis of variance (ANOVA). In order to quantify the strength of the relationship between the variables a linear Pearson's correlation was determined. The correlation coefficients were calculated using the statistical package SPSS for Windows (v15.0, SPSS, Chicago, Illinois, USA), and results were recognised as statistically significant at $p \leq 0.05$.

3. Results and Discussion

3.1. Relationship Between Acoustic Screening Method and HPLC

In the analysed wheat samples using HPLC-TOF-HRMS, three DON conjugates were obtained: D3G, 15-ADON, and 3-ADON. A relatively large difference in DON levels were noticed for the single DON conjugates. In the sample of wheat 6 (W6) with highest DON amount (1330 μg/kg), D3G content was six times lower in than DON, followed by 15-ADON (178 μg/kg), while 3-ADON contained the least (34 μg/kg). The majority of the less contaminated wheat samples contained significantly lower amounts of DON conjugates compared with their free form.

198 The acoustic technique, validated on a single laboratory as previously reported
199 (Juodeikiene et al., 2014), with the selected optimal frequency of 32.6 kHz, was applied for the
200 initial DON content analysis of contaminated wheat grains. A strong linear relationship
201 between DON (Figure 1A), as well as D3G (Figure 1B), content, measured by HPLC-TOF-
202 HRMS, and the amplitude of the acoustic signal (Ap) in the various contaminated wheat
203 samples (R^2 = 0.85; 0.82, $p < 0.05$, respectively) was obtained.

204 This study confirmed that DON conjugates co-occur with its free forms in wheat grains.
205 DON is the parent mycotoxin formed in the plant by fungi and masked toxins are the
206 metabolites of the DON (EFSA, 2014). The most studied masked toxin D3G is the phase II
207 metabolite of DON and it is less toxic than parent mycotoxin. However, it can be reversed back
208 to DON by human colonic microbiota (Jin et el., 2018). Up till now the level of D3G is not
209 regulated in cereal and its products. Therefore, the ratio of D3G/DON should be taken into
210 consideration because D3G can be converted to DON and reactive its toxicity (Simsek et al.,
211 2013). In our studies, the D3G/DON ratio in analysed wheat samples were between 17% and
212 30%. These results are in agreement with other authors, showing that D3G in wheat has been
213 found at relative proportions of 20–70% of free DON (Guo et al., 2020). Several studies
214 indicated that the level of D3G contamination reached half that of DON in cereals and cereal
215 by-products. D3G often co-occur with DON in crops, cereal-based food, and feed products in
216 high proportions (up to 100%) and at concentrations ranging from 2–1700 µg/kg (Berthiller et
217 al., 2005b; Hajslova, 2008; Sasanya et al., 2008; Kostelanska et al., 2011; Vendl et al., 2010;
218 Desmarchelier & Seefelder, 2011; Li et al., 2011; Malachova et al., 2011; De Boevre et al.,
219 2012; Berthiller et al., 2013; Simsek et al., 2013). The ratio of D3G/DON reached 20% in
220 grains and even exceeded 100% after processing (Berthiller et al., 2005a; Berthiller et al., 2013;
221 Varga at al., 2013).

The ratio of D3G/DON varied between the level of DON contamination of wheat samples. The results indicate that samples of wheat (W1-W5) with lower DON levels (68.8-786 µg/kg) had higher D3G/DON ratio (on average by 22.7%) and sample (W6) with highest DON level (1330 µg/kg) had lower D3G/DON ratio (on average by 16.7%). These results are in agreement with findings of Lemmes et al. (2016) who reported that the amount of D3G is relative to DON contamination and samples with the lowest DON contamination show the highest level of D3G. Whereas this masked toxin is hazardous to human and animal health , the levels of D3G should be taken into account because if undetected it could cause risk in food/feed safety especially in the products contaminated with lower level of DON or near the maximum allowed limit.

Obtained acoustic results are in agreement with previous studies, which indicated strong relationship between the amount of scabby kernels and DON content in wheat (Juodeikiene et al., 2014). Fungal damaged grains become more porous and shrivel (in case of wheat they become scabby). For corn kernels, which were affected by *A. flavus* shrivelling is less prevalent in comparison to wheat grains because corn kernel pericarp is sturdier (Juodeikiene et al., 2020). Upon harvest, a mixture of healthy and shrivelled grains (or more porous kernels) occurs. As can be observed form other experiments, the terms of air-flow resistivity, porosity and tortuosity describes the acoustic behaviour of porous granular media. Furthermore, it was found that the mechanism of sound absorption significantly depends on particle size and shape in beads of cereal grains (Guo et al., 2005). Therefore, it is advisable to use developed equipment during the harvesting, where usually dominates one genotype of cereal (with same particle size and shape). This study showed additional possibilities of acoustic sensors for screening DON conjugates, as well.

So far, country-specific emergence data on masked mycotoxins on a global scale is very limited and causes a final risk assessment impossible. In order to achieve unequivocal determination of the mycotoxicological load in food and feedstuff, continuous global surveys

of masked mycotoxins are imperative. Therefore, the application of a rapid method for masked toxin screening is highly important.

3.2 The Effect of LAB Fermentation on the biological detoxification of Fusarium spp.-Contaminated Wheat Grains

Alternative biotechnological means for detoxification of *Fusarium* spp. contaminated cereals and mycotoxin reduction are still under research. Lately, considerable interest in the application of LAB strains with antimicrobial activity for the bioconversion of cereal biomasses to more safety products has surfaced, which could by applied for food and feed preparation. The effect of biological treatment on the growth of LAB and pH changes under different contamination levels in the media is an essential point for the mechanism of mycotoxin degradation.

In this study, evaluation of LAB viability on contaminated *Fusarium* spp. wheat grains has been considered. Microbiological analysis of the fermented wheat grains with different contamination revealed that externally added cultures as well as contamination of raw material affects the process of fermentation (Table 2). A great reduction in LAB count from 8.50 to 7.61 $\log_{10}$ CFU/g was measured with *L. cas*ei in samples with low contamination (DON >69 µg/kg), respectively, compared to the sample with high DON content (<1330µg/kg). *L. plantarum* and *L. paracasei* have not shown significant response to medium contamination. The lowest sensitivity for contamination was observed with *L. uvarum*, showing an increase in multiplication from 8.77 (68.8 µg/kg DON) to 9.99 (1330 µg/kg DON).

In this study, some fermented samples contained LAB cells within ranges reported by other authors (Rizzello et al., 2019, Xu et al., 2019). The slower growth of this LAB strain in the contaminated medium could have a negative effect on the formation of organic acids. However,

most LAB strains, such as *L. uvarum, L. paracasei,* and *L. plantarum,* remain active in contaminated grain samples and accumulated well in organic acids by reducing the medium pH value to <3.5. Though, for the initial sourdough fermentation stage, the pH was within values in average 6.00 (Table 2).

Antimicrobial LAB occurs as starter cultures and as a part of the natural microbial population during fermentation process in the food/feed industry. One of the most preferred properties of LAB as a starter culture for fermented product processes is quick acid production (Şimşek et al., 2006; Clarke et al., 2002). Antimicrobial activity of LAB is related to its low pH and production of organic acids, such as lactic, sorbic, formic, propionic and benzoic acids. In fact, the metabolites produced by antimicrobial *lactobacillus* strains were pH-dependent (Rizzello et al., 2011).

Research during the past decades has demonstrated that there is a synergistic effect among combinations of organic acids or organic acids combined with proteinaceous compounds (Corsetti et al. 2007). The antifungal activity of *Lactobacillus casei* AST18 demonstrated a synergistic effect with lactic acid and the cyclopeptides (Li et al., 2012), explaining the broad-spectrum antifungal ability of this LAB strain against fungi and yeasts in food and feed (Ström et al., 2002).

Efficiency of the fermentation process was analysed by qualitative and quantitative composition of DON and its conjugates using different LAB strains, as well as wheat grains with different contaminations (Figure 2A–D). Regression analysis was carried out for all the strains, in order to characterize their bioconversion ability after 24 hours of fermentation under optimal conditions.

The results obtained in quantitative DON analysis confirmed the biotransformation degree (qualified as the average amount of DON) of the fermented material to be lower by 50% compared to the reference sample (control; Figure 2A). The application of fermentation

297 resulted in lower amounts of DON by 56.7% in highly contaminated grain samples (1330 µg/kg
298 DON) compared to the bio-treated lower contaminated grain sample with prevailing amounts
299 of DON (<800 µg/kg). According to the obtained results, a significant reduction in DON by 4,
300 2.4, 3, and 1.65 times, respectively, using fermentation can be achieved with the application of
301 *L. uvarum* at different cereal contamination levels (1330, 786, 504, and 314 µg/kg of DON).
302 With the application of *L. casei*, *L. plantarum*, and *L. paracasei*, the amount of DON in the
303 highly contaminated (1330 µg/kg) fermented samples decreased by 42%, 56.6%, and 53.1%
304 compared to the control samples with wheat material but no bio-treatment.
305 Qualitative analysis of masked trichothecene revealed a significant reduction of D3G, 3-
306 ADON, and 15-ADON in the LAB-fermented material samples. The amount of D3G in the
307 fermented samples decreased, on average, by 84.1% (Figure 2B) compared to the control
308 samples without bio-treatment. Furthermore, other DON conjugates, such as 15-ADON and 3-
309 ADON, were not identified, respectively, in the *L. uvarum* and *L. brevis* and *L. casei* and *L.*
310 *paracasei* fermented samples (Figure 2C, D). According to the obtained results, most of the
311 strains were able to remove several masked toxins, but considerable differences among these
312 strains were still observed. According to Muhialdin et al. (2020), detoxification level of LAB
313 depends on the density and viability of LAB cell as well as pH of the medium. These
314 characteristics varied significantly between the LAB strains. The response of LAB strains to
315 the level of the medium contamination is different between the microorganisms. This
316 phenomenon has been proved in this study by testing the LAB grow intensities on tested
317 samples.
318 The obtained results are in agreement with studies from (Niderkorn et al. 2006), which
319 described the abilities of twenty-nine LAB strains, including *L. rhamnosus* strain GG, to
320 remove mycotoxins (such as DON and fumonisins B1 and B2) in sub-acidic medium. Up to
321 55% removal was achieved for DON by *Lactobacillus delbruekii* ssp. *bulgaricus*, while

removal by *L. rhamnosus* strain GG was around 54% (Niderkorn et al., 2006). Trichothecenes, such as DON, 3-ADON, nivalenol (NIV), diacetoxyscirpenol (DAS), fusarenon (FX), T-2 toxin (T-2), and HT-2 toxin (HT-2), elimination possibilities were analysed by (El-Nezami et al., 2002). The obtained results confirmed that *Lb. rhamnosus* GG, *Lb. rhamnosus LC-705*, and *Propionibacterium freudenreichii ssp. shermanii* JS were able to trap seven trichothecenes from liquid media. The authors demonstrated that the efficiency of LAB to remove trichothecenes (20 µg/ml) varied significantly, depending on the toxins and LAB strains considered. The most effective strain showed the ability to bind four of the seven toxins tested, as no degradation products were detected. The percentage of bound toxins varied between 18% and 93% (El-Nezami et al., 2002). None of the LAB was capable of binding 3-ADON. The novelty of this research is that fermentation with *L. uvarum* strain proofed a very positive effect on elimination of DON as well as its masked toxins from the wheat raw material.

According to reviewed research, the biological elimination of mycotoxins in fermentation media can be achieved using several mechanisms such as by absorption of viable LAB cells or mycotoxins degradation could be caused by LAB produced enzymes and obtained metabolites (Muhialdin et al., 2020; Zadeike et el., 2021).

Enzymatic activities of fungi in contaminated raw cereal also could play an important role in the degradation mechanism of mycotoxins during LAB fermentation, converting them into non-toxic compounds. Therefore, in the next stage of the experiment, the enzymatic profiles of contaminated raw wheat samples were investigated and their relationship with mycotoxins has been taken into considerations.

3.3 Biochemical Changes Related to Mycotoxin Degradation During LAB Fermentation and the Reduction of DON and its Conjugates

At this stage of the experiment the enzymatic activities (amylolytic, endoxylanolytic and proteolytic) of contaminated wheat samples were determined before fermentation. The results of enzymatic activity studies in grain raw material with different contamination and are presented in Table 3.

Obtained results demonstrates that with the increase of DON and D3G in wheat grains the activity of amylases also increases, showing a very strong positive relationship between these mycotoxins and amylolytic activities ($R^2 = 0.8235$, $R^2 = 0.9314$, respectively). Amylase activity increased on average by 20% with increasing 3ADON in wheat samples and a weak correlation was observed between the analyzed parameters ($R^2 = 0.3559$). No statistically significant change was observed between 15ADON and amylase activity ($R^2 = 0.1135$). Additionally, the same tendency was observed that with increasing concentration of mycotoxin contamination, xylanase activity in the studied samples also increased. A very strong positive correlation was found between DON and D3G mycotoxin content in wheat and xylanase activity ($R^2 = 0.8694$; $R^2 = 0.9937$, respectively). Meanwhile, a moderate positive relationship was found between 3-ADON and xylanase activity ($R^2 = 0.4879$). The effect of 15-ADON-producing fungi on xylanase activity was found to be insignificant ($R^2 = 0.0513$).

An increase in proteolytic activity was also observed with increasing mycotoxin levels in the samples. Samples with the highest DON and D3G levels indicated the highest proteolytic activity. However, weak direct relationship was found between these mycotoxins (DON and D3G) and proteolytic activity ($R^2 = 0.3948$; $R^2 = 0.4311$, respectively). Meanwhile, microscopic fungi producing other mycotoxins (3-ADON and 15-ADON) are not thought to have protease activity ($R^2 = 0.0734$; $R^2 = 0.3249$, respectively). In all cases, higher enzymatic activities were observed in the samples with higher levels of DON and D3G contamination.

On the basis of the aforementioned microorganism, we assumed that some active enzymes of fungi may be also responsible for the degradation of DON. According to the

literature, fungi is a source of enzymes that are capable of converting mycotoxins into less toxic or non-toxic products and have the potential to be used to increase the safety of agricultural products or as additives in feed production (Li et al., 2020). The enzymes specifically exert a degradation effect on DON by destroying its structure and resulting in toxic and nontoxic metabolites (Juodeikiene et al., 2012). Furthermore, our results show that the highest decontamination level was achieved by application of *L. uvarum* strain, which was the least sensitive for contamination in the media and showed the ability to multiply most effectively during the 24h fermentation process. This phenomenon could explain that the mechanism of mycotoxins reduction could be related to absorption by viable LAB cells. Regarding pH, significant differences have been found between this factor and fungi/mould growth, as well as DON and its conjugates in contaminated wheat samples. The pH effect was shown to be related to many factors, such as substrate, mould strains, incubation temperature, incubation period, and the occurrence of competing microflora (Gourama & Bullerman, 1995). LAB strains, such as *L. uvarum*, demonstrated highest grow activity and pH changes during fermentation; the elimination of toxins was also most efficient. The reduction of pH in the media shows that reduction of *Fusarium* mycotoxins in contaminated wheat could be also caused by LAB enzymes and obtained metabolites.

Most probably, mycotoxin elimination includes a combination of microbial conjugation and enzymatic degradation, both of which can be achieved by biological systems. However, the mechanism and products of degradation or conjugation have yet to be evaluated.

This study confirmed, that biological detoxification of mycotoxins is an alternative strategy in sustainable food chain with a possibility to reduce the contamination in food/feed, increase the safety of the products and to avoid economical losses. Our findings indicate that the microorganisms are playing important role in the elimination of mycotoxins in contaminated wheat and traditional fermentation could be improved by using selected LAB

strains, e.g. *L. uvarum*, in order to significantly reduce contamination in raw material. This strategy could be applied for the development of fermented products in food/feed industry (Muhialdin et al., 2020). Moreover, the combination of LAB strains with different enzymatic approaches for decontamination of wheat samples could be developed, as well.

4. Conclusions

Crop contamination is global issue in food safety and the most found contaminant in wheat is deoxynivalenol (DON). Our findings on the strategy of reducing *Fusarium* spp. mycotoxins in wheat grain shows that biological detoxification can be achieved by application of fermentation using LAB strains with antimicrobial activity which successfully reduces or eliminates DON and its conjugates (D3G, 3-ADON and 15-ADON) from the media.

The level of decontamination of the samples depends on the LAB strain used for bio-treatment. The most effective reduction of DON and D3G as well as elimination of conjugates such as 3-ADON and 15-ADON indicated *L. uvarum* strain which was the least sensitive for contaminated media and maintained to multiply effectively.

Our study demonstrate that amylolytic and xylanolytic activities strongly correlate with DON (R^2 = 0.8235, R^2 = 0.8694, respectively) and D3G contents (R^2 = 0.9314, R^2 = 0.9937, respectively), thus the mechanism of mycotoxin degradation in wheat grain could be related not only with microbial conjugation but also with enzymatic degradation of mycotoxins. Consequently, future research is needed to evaluate the products of degradation or conjugation.

These findings in biological mycotoxin elimination, separately or in combination with acoustic screening of raw material, lead themselves to increase cereal processing efficiency and sustainability in the food and feed production chain. The application of rapid methods, such as broadband capacitive acoustic sensors, are still very attractive solutions for *Fusarium* mycotoxins prevention in wheat grains. High correlations were obtained between acoustic

screening method and HPLC for DON and D3G (R^2 = 0.85, R^2 = 0.82, respectively). We conclude that this acoustic penetration spectrometer is wider-ranging and can be applied not only for DON monitoring and also for D3G conjugate.

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Figure captions:

Figure 1. Relationship between DON (A) and D3G (B) contents in wheat grain samples and acoustic signal amplitude.

Figure 2. The effect of fermentation using antimicrobial LAB (*L. casei, L. paracasei, L. plantarum*, and *L. uvarum*) on DON (A), D3G (B), 3-ADON (C), and 15-ADON (D) content in different *Fusarium* spp.-contaminated wheat samples (W1–W6).

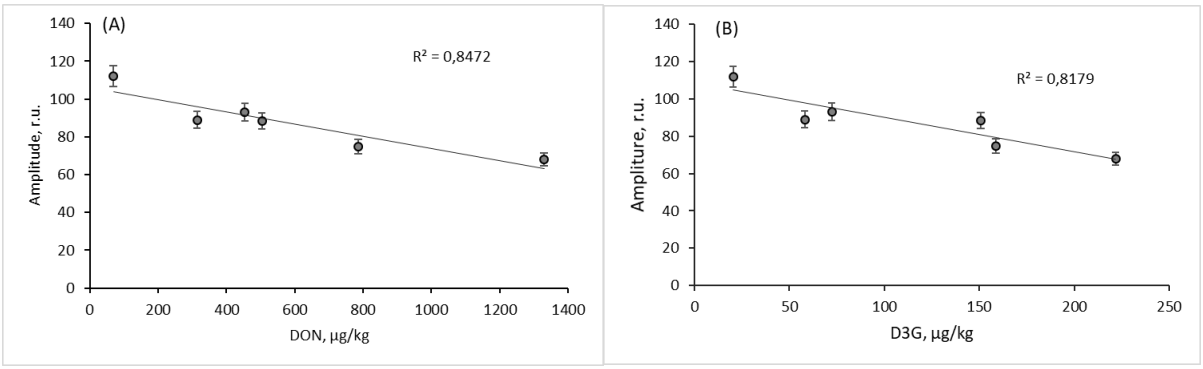

Figure 1.

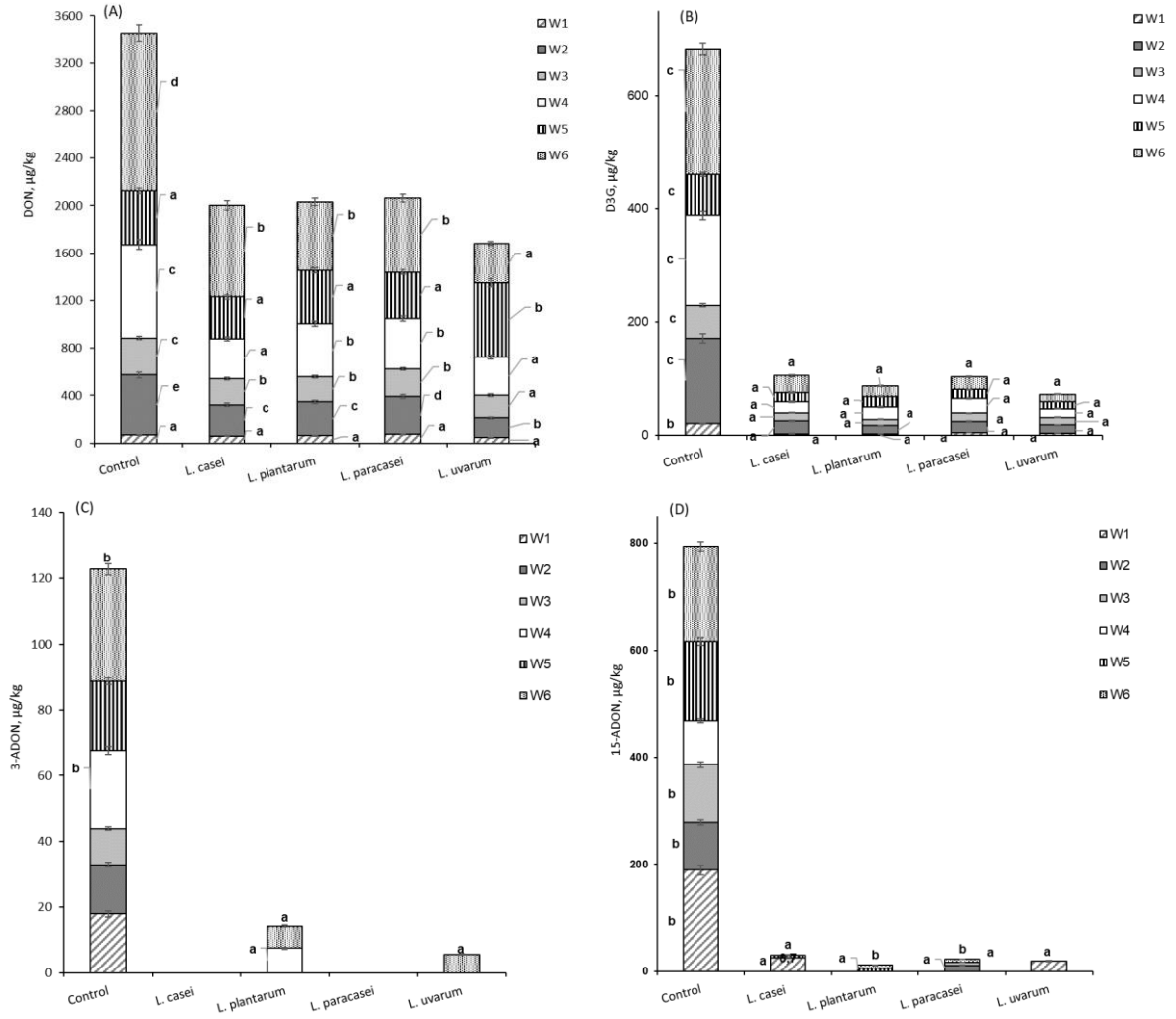

Figure 2.

Table 1
The levels (µg/kg d.m.) of mycotoxins detected by HPLC-TOF-HRMS in wheat samples.

Samples	DON, µg/kg	D3G, µg/kg	3-ADON, µg/kg	15-ADON, µg/kg
W1	68,8	20,4	18	189
W2	504	151	14,9	89,5
W3	314	58,1	11	108
W4	786	159	23,8	81,6
W5	453	72,3	21	148
W6	1330	222	34	178

W - wheat sample; DON – deoxynivalenol; D3G - deoxynivalenol-3-glucoside; 3-ADON - 3-acetyldeoxynivalenol and 15-ADON - 15-acetyldeoxynivalenol; Results are presented as means ± SD reported in dry basis (n = 3).

Table 2
Influence of 24 h fermentation with *L. casei*, *L. plantarum*, *L. paracasei*, and *L. uvarum* strains in different contaminated (DON 68.8–1330) wheat grain samples (W1-W6).

Samples		pH	LAB log_{10} CFU g^{-1}	YMC log_{10} CFU g^{-1}
Control	W1	5.99 ± 0.08	-	2.48 ± 0.03
	W2	6.04 ± 0.06	-	2.70 ± 0.12
	W3	5.93 ± 0.10	-	2.30 ± 0.09
	W4	6.00 ± 0.09	-	2.00 ± 0.10
	W5	6.02 ± 0.07	-	2.00 ± 0.11
	W6	6.01 ± 0.08	-	3.08 ± 0.15
L. casei	W1	3.47 ± 0.13	8.50 ± 0.05	4.69 ± 0.08
	W2	4.01 ± 0.09	8.05 ± 0.03	5.89 ± 0.05
	W3	3.71 ± 0.10	8.02 ± 0.09	5.36 ± 0.10
	W4	3.91 ± 0.11	7.68 ± 0.07	6.02 ± 0.11
	W5	3.57 ± 0.04	7.73 ± 0.10	5.65 ± 0.09
	W6	3.63 ± 0.10	7.61 ± 0.08	6.34 ± 0.07
L. plantarum	W1	3.41 ± 0.16	8.75 ± 0.15	4.39 ± 0.10
	W2	3.38 ± 0.01	9.02 ± 0.12	4.12 ± 0.11
	W3	3.37 ± 0.13	8.92 ± 0.09	4.63 ± 0.09
	W4	3.48 ± 0.12	8.83 ± 0.11	4.48 ± 0.10
	W5	3.50 ± 0.07	8.24 ± 0.13	5.16 ± 0.11
	W6	3.49 ± 0.09	9.17 ± 0.10	4.23 ± 0.10
L. paracasei	W1	3.44 ± 0.12	8.40 ± 0.15	4.46 ± 0.12
	W2	3.46 ± 0.09	8.65 ± 0.09	5.38 ± 0.10
	W3	3.43 ± 0.11	8.34 ± 0.10	5.34 ± 0.09
	W4	3.57 ± 0.04	8.77 ± 0.19	5.98 ± 0.10
	W5	3.38 ± 0.08	8.50 ± 0.20	4.87 ± 0.07
	W6	3.50 ± 0.10	8.80 ± 0.11	5.12 ± 0.11
L. uvarum	W1	3.38 ± 0.15	8.77 ± 0.03	4.16 ± 0.09
	W2	3.24 ± 0.09	9.70 ± 0.10	3.99 ± 0.05
	W3	3.25 ± 0.08	9.97 ± 0.09	4.06 ± 0.07
	W4	3.41 ± 0.10	9.32 ± 0.11	3.97 ± 0.08
	W5	3.27 ± 0.12	8.75 ± 0.13	4.57 ± 0.10
	W6	3.45 ± 0.06	9.99 ± 0.08	3.23 ± 0.08

Control – initial wheat samples (before fermentation).
LAB – lactic acid bacteria count.
YMC – yeast and mould count.
Data expressed as mean values (n = 5) ± SD

Table 3
Enzymatic profile of *Fusarium* spp. contaminated wheat samples at the initial stage of the experiment.

Samples	Amylolytic acitivity, AU/g	Xylanolytic activity, KU/g	Proteolytic activity, PU/g
W1	203.25 ± 4^{a}	98.297 ± 3^{a}	142.14 ± 11^{a}
W2	408.40 ± 11^{b}	135.531 ± 4^{a}	179.67 ± 6^{b}
W3	315.87 ± 6^{a}	109.805 ± 6^{a}	187.95 ± 5^{b}
W4	411.14 ± 12^{b}	141.652 ± 3^{a}	183.87 ± 6^{b}
W5	333.95 ± 16^{a}	110.529 ± 6^{a}	189.08 ± 3^{b}
W6	478.84 ± 11^{c}	157.343 ± 1^{b}	186.34 ± 2^{b}

W - wheat sample; Results are presented as mean values ± SD (n = 3). $^{a–c}$ Means within a column with different superscript letters are significantly different ($p < 0.05$);

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Grazina Juodeikiene: Conceptualization, Methodology, Data curation, Writing- Original draft preparation**. Karolina Traksleyte-Rupsiene**: Investigation, Writing- Original draft preparation. **Elizabet Janić Hajnal**: Investigation, Resources. **Vadims Bartkevics, Iveta Pugajeva, Dovile Klupsaite, Darius Cernauskas*:*** Formal Analysis. **Vita Lele**: Resources. **Daiva Zadeike:** Writing- Reviewing and Editing. **Elena Bartkiene**: Metodology.