DARIUS ČERNAUSKAS

# DETECTION AND DETOXIFICATION OF FUSARIUM SPP. CONTAMINATED CEREAL GRAINS FOR MALT PRODUCTION BY ANTIFUNGAL BIOAGENTS

SUMMARY OF DOCTORAL DISSERTATION

TECHNOLOGICAL SCIENCES, CHEMICAL ENGINEERING (05T)

Kaunas
2017

KAUNAS UNIVERSITY OF TECHNOLOGY

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KAUNO TECHNOLOGIJOS UNIVERSITETAS

DARIUS ČERNAUSKAS

# *FUSARIUM* SPP. PAŽEISTŲ JAVŲ GRŪDŲ SALYKLO GAMYBOJE APTIKIMAS IR DETOKSIKACIJA ANTIMIKROBINĖMIS BIOPRIEMONĖMIS

Daktaro disertacijos santrauka
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## ABBREVIATIONS

| | |
|---|---|
| DDGS | dried distiller's grains with solubles |
| DON | deoxinivalenol |
| ELISA | enzyme-linked immunosorbent assay |
| FAO | the Food and Agriculture Organization of the United Nations |
| GC | gas chromatography |
| HPLC | high-performance liquid chromatography |
| kDa | kilodalton |
| LAB | lactic acid bacteria |
| Lb | *Lactobacillus bulagircus* |
| LoD | limit of detection |
| LoQ | limit of quantitation |
| Ls | *Lactobacillus sakei* |
| MRS | de Man, Rogosa and Sharpe |
| MS | mass spectrometry |
| MS/MS | tandem mass spectrometry |
| PAGE | polyacrylamide gel electrophoresis |
| Pa7 | *Pediococcus acidilactici* |
| Pp8 | *Pediococcus pentosaceus* |
| Pp9 | *Pediococcus pentosaceus* |
| Pp10 | *Pediococcus pentosaceus* |
| $R^2$ | the coefficient of determination |
| R% | means of recovery |
| RSD | relative standard deviation |
| T-2/HT-2 | T-2/HT-2 mycotoxins |
| TRIS | 2-amino-2-hydroxymethyl-propane-1,3-diol |
| UHPLC | ultra high-performance liquid chromatography |
| ZEA | zearalenone |

### List of microorganisms genera

*A. – Aspergillus*
*C. – Claviceps*
*F. – Fusarium*
*K. – Kluyveromyces*
*L. – Lactobacillus*
*N. – Neotyphodium*
*P. – Pediococcus*
*Penic. – Penicillium*
*Pith. – Pithomyces*
*S. – Saccharomyces*

# 1. INTRODUCTION

**Motivation for the research.** Cereals are a significant and important resource in human food as well as livestock feed worldwide. Grain quality assurance must be controlled by the entire grain production and processing chain from the primary cereal grain production to product marketing to the consumer. Particular attention is paid to contaminants – fungi originating from grain cultivation and storage. Mycotoxins-producing fungi disrupt the grains by changing their structure; they are able to produce toxins which contaminate human food and animal feed.

One of the basic raw materials for malt production are malting cereals (barley and wheat), the quality of which is of primary significance. The malting industry requires malt with a high extract yield, high levels of enzyme activity, and good modification to manufacture consumer products of excellent quality. Optimal germination performance, such as germination capacity, is definitely one of the most important quality criteria for malting grains.

FAO analysis showed that annually up to 25 % of the produced grain can be contaminated with mycotoxins. For this reason, mycotoxins may cause huge economic losses; they represent a significant hazard to the cereal processing chain especially at the time of flowering and storage when respectively the weather and temperature/ humidity may be out of range. During the last decades, *Fusarium graminearum* has become the dominant species causing scab or head blight of cereals in the world. Global warming and therefore the change towards higher temperatures/humidity favors its dominance in the disease complex fostering the occurrence of mycotoxins such as trichothecene-group mycotoxin deoxynivalenol.

*Fusarium* spp. could have a more prominent negative impact on the malting grain sprouting capacity when seeds are stored for a longer time. Seed distributors should analyze each lot for the presence of microbial pathogens by using internationally accepted analytical methods and keeping up-to-date agricultural records in order to eliminate contaminated seeds. Unfortunately, these methods are prohibitively costly. Therefore, consumer protection against mycotoxins is necessary to feature efficient control of their determination in the grain processing line. Worldwide, the common practice of determining mycotoxins is the use of labor- and time-intensive fundamental chemical, physical and enzyme immunoassay analysis. Considering the fact that the detection methods of these mycotoxins are complex and expensive, special attention should be paid to any innovative mycotoxin determination technology which would allow quick and inexpensive detection of mycotoxins in the raw materials.

Fungal infection not only results in the accumulation of mycotoxins but also causes grains to shrivel and become more porous. This phenomenon is

known as head blight or scab, and is widely seen as one of the indicators of poor grain quality. Due to changes in the grain microstructure, a rapid and non-destructive method of the evaluation of the quality and safety of grains is therefore required so that to detect and subsequently eliminate these toxins from the food chain. In the framework of EUREKA ITEA2 project ACOUSTICS, the first portable acoustic device predicting the level of deoxynivalenol (DON) in cereal grains has been developed by using a broadband capacitive ultrasonic transducer. The project results showed that the acoustics method, applied for grain safety monitoring for the first time, is thus innovative and important in terms of ensuring the safety of grains. However, to the best of our knowledge, so far, no studies of the influence of *Fusarium* spp. and their metabolites on the chemical composition and technological properties of malting grain have been performed. On the other hand, it is fundamentally important to ensure appropriate malting grain storage conditions. When stored grains are contaminated, they are starting to breathe, the grain silos temperature increases, and grains consequently begin to produce $CO_2$ gas. It is believed that $CO_2$ monitoring allows accurate detection of the cereal contamination as well as much earlier discovery of the grain storage silos and insect infection than the temperature and humidity monitoring methodology. In order to increase the precision of the method and to extend the field of application of grain storage control in non-ventilated silos, it is appropriate to devise new technical solutions to monitor $CO_2$ levels in the grain mass.

Cereal processing which may involve physical, chemical or microbiological decontamination can often be effective in destroying or redistributing *Fusarium* mycotoxins. In recent years, biological decontamination and biodegradation of mycotoxins with microorganisms or enzymes has been used more commonly. The essence of the biological decontamination methodology is the use of microorganisms inhibiting the biosynthesis of mycotoxins, by isolating (or ‘binding’) mycotoxins and suppressing or transforming them into non-toxic or less toxic compounds. One promising and economically attractive strategy is the treatment of malting seeds with anti-fungal LAB bio-products (*Bioekotech* project), which could be performed seeking to decontaminate a wide range of plant seeds as well as malting grains. It should be noted that Lithuania is rapidly expanding in the field of production of biofuels (e.g. bioethanol, biodiesel). The efficiency of bioethanol production from cereal biomass can be increased by selecting LAB in combination with a biocatalyst. Furthermore, biotechnological treatment may be considered to have a positive effect on the residue of alcohol fermentation (dried distillers grains with solubles (DDGS)) as feed.

Therefore, by using LABs in fermentation processes and creating new bioproducts, e.g. for the treatment of malting grains, an additional study of the influence of bioproducts on the germination criteria is required. The creation of

such a type of bioproducts is necessary in order to increase the sprouting capacity of barley grains by eliminating the development of phytopathogens such as fungi producing secondary metabolites (e.g. mycotoxins) during the sprouting of the malting grains as well as in order to increase the efficiency of malt production.

The bioproducts used for the processing of malting grain seeds in organic farming should be not only effective but also cheap. Upon setting such a goal, the search for new media for the cultivation of LAB is relevant by paying attention to lactose by-products (whey permeate) of the dairy industry which could reduce the price of the bioproduct manufacturing.

It is also important to determine the effect of LAB biotreatment on the biochemical changes of the malting grain protein. The proteomic comparison of seeds under normal and stressed conditions could identify useful markers serving this purpose. Such environmentally beneficial plant-protection strategies devised in the course of molecular analysis of developing cereal grains could be easily integrated into sustainable agricultural practices in order to increase yields, safety and quality without incurring environmental harm.

Alternative methods of mycotoxin detoxification in malting grains involve the use of ozone ($O_3$). Ozone, a powerful oxidant, possesses numerous beneficial applications and is very familiar within the food processing industry. It has long been used in food processing as a water treatment agent disinfecting and eliminating odors, taste and color. The rapid decomposition of ozone ($O_3$) residues to oxygen ensures that ozone leaves no chemical residues when used in food treatment. It is authorized as a safe antimicrobial agent that can be applied to foods directly or through aqueous phases for the destruction and/ or detoxification of mycotoxins in malting grains. However, the antimicrobial effectiveness of ozone depends on several factors – the applied amount and various environmental factors, such as grain mass temperature, humidity and surface properties. It is thus important to evaluate the balance between the efficiency and safety concerns of ozone in malting cereal, its storage and procession.

**The aim of the thesis**. Development of fast and efficient methods for the detection of *Fusarium* spp. contaminated malting cereal grain and assessment of possibilities of detoxification of *Fusarium* spp.-contaminated cereal grains with such antifungal bioagents as LAB bioproducts and ozone.

**The following tasks were set in order to achieve the aim of the thesis:**

1. To determine the connection between the concentration of mycotoxin DON produced by *Fusarium* spp. and the chemical composition and microstructure of contaminated malting grains;
2. To determine the connection between the level of contamination of mycotoxin DON produced by *Fusarium* spp. experienced by malting

wheat grains and the range of acoustic signal parameter values;

3. To evaluate innovative technical solutions allowing to monitor the changes of $CO_2$ concentration levels in wheat grain mass contaminated with *Fusarium* spp;
4. To evaluate the influence of ozone on the reduction of mycotoxins in malting wheat grains;
5. To assess the influence of LAB bioproducts and cultivated cheese whey permeate nutritional medium on the volume of the resulting metabolism products, increase of efficiency of malt production and decrease of mycotoxin concentration in malting wheat grains;
6. To identify the effect of LAB on the quantitative composition of the protein substances of malting wheat grain contaminated with *Fusarium* spp. at the time of grain germination by performing protein fractionation according to molecular mass quantitative composition affecting LAB biotreatment stress resistance during *Fusarium* spp. contaminated wheat seed germination;
7. To evaluate the use of various biotechnological means – LAB and grain enzymatic hydrolysis on the increase of the efficiency of *Fusarium* spp. contaminated barley grain alcohol fermentation and the reduction of the amount of mycotoxins in the production waste (distiller's dried grains with solubles).

**Novelty of the study.** For the first time, a new acoustic spectrometer is tested and adapted for fast and non-contact determination of the mycotoxin deoxynivalenol (DON) in malting wheat grains affected by *Fusarium* spp.

For the first time, the effect of LAB bioproduct and ozonation on mycotoxin reduction in malting wheat grains is tested.

For the first time, the effect of LAB bioproduct cultivated in cheese dairies whey permeate medium on mycotoxin reduction in malting wheat grains is tested.

For the first time, the influence of LAB biotreatment during grain germination on *Fusarium* spp. is tested, and the quantitative composition of contaminated malting grain protein substances is determined;

**The practical value of the work.** A new acoustic technique for quick and non-contact determination of mycotoxin deoxynivalenol (DON) was developed in malting wheat contaminated by *Fusarium* spp.

Bioproducts of LAB intended to process organic wheat grain seeds were developed. The new bioproducts allow to reduce DON mycotoxin in malting grains and to increase the germination energy of the contaminated malting wheat.

It was discovered that ozone allows reducing other trichothecenes in addition to DON, such as ZEA, T-2 and HT-2 mycotoxin in contaminated malting grains.

LAB, enzymes and yeast used for *Fusarium* spp. damaged barley grain

during fermentation increase the yield of bioethanol and the reduction of DON in DDGS.

**Defended claims of the dissertation.**

1. A new acoustic technique is capable of quickly and accurately detecting contamination in malting wheat grains, which correlates with mycotoxin DON produced by *Fusarium* spp;
2. The LAB bioproducts reduce the DON concentration and fungi infection on the surface of contaminated wheat grain seeds and increase the grain germination energy;
3. LAB, enzymatic preparations and yeast used during the fermentation process of barley raw materials contaminated with *Fusarium* spp. increase the yield of bio-ethanol and decrease the concentration of *Fusarium* spp.-produced mycotoxin DON in DDGS.
4. Ozone has a detoxification effect on the trichothecenes DON, ZEA, T-2 and HT-2 contaminated with *Fusarium* spp.

**Structure and content of the dissertation.** The thesis is written in the Lithuanian language. It consists of a list of abbreviations, an introduction, a literature review, materials and methods description, results presentation and discussion, conclusions, a list of references, and a list of publications relevant to the subject of the dissertation. The dissertation text covers 113 pages, it features 24 tables and 40 figures. The list of references includes 253 bibliographic sources.

**Approval and publications of the results of the study.** The results of the research have been published in 4 scientific articles in the journals indexed by Thomson Reuters (WOS) and in proceedings books of 9 international conferences. The results of the research have also been presented in 5 international scientific conferences and in 2 journals of Lithuanian scientific conferences.

## 2. RESEARCH OBJECTS AND METHODS

### 2.1. Research objects

**Contaminated seeds.** Wheat grains (harvested in 2012, milling company Kauno Grūdai, Kaunas, Lithuania), scabby wheat grains contaminated with DON (harvested in 2011, breeding and seed company *Florimond Desprez*, Cappelle-en-Pévèle, France) and samples of Lithuanian barley grain (harvested in 2010, obtained from different farms of Lithuania) were used for the analysis of the chemical composition and the investigation of qualitative characteristics.

The following wheat grain samples were used for acoustic analysis: the model systems were created by mixing wholesome wheat grains (harvested in

2012, moisture content 13.5 %; milling company *Kauno Grūdai*, Kaunas, Lithuania) and scabby wheat grains contaminated with 4000 µg/kg DON (harvested in 2011, moisture content 13.8 %; breeding and seed company *Florimond Desprez*, Cappelle-en-Pévèle, France) in different proportions, ranging from 10 % to 100 % contaminated grains in 10 % steps (11 test samples in total, including a wholesome grain sample). Each sample weighed 200 g. In order to test the method in practice, 34 naturally-contaminated soft wheat grain samples harvested in 2013 with a moisture content of 13–14 % and DON concentrations of 254–1600 µg/kg from the Lithuanian milling company *Kauno Grūdai* and the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry (LRCAF-IA), were used.

Wheat grains (harvested in 2012, milling company *Kauno Grūdai*, Kaunas, Lithuania) and scabby wheat grains contaminated with DON (harvested in 2011, breeding and seed company *Florimond Desprez*, Cappelle-en-Pévèle, France) were used for $CO_2$ detection analysis.

Contaminated grains of spring wheat cultivars Arktis LEU 60210 (protein content 11–11.5 %) grown and harvested in 2012 (contaminated with *Fusarium* spp., the dominant species being *F. culmorum*) were collected from various farms in Lithuania (Plungė District Municipality) and were used for LAB bio-products regarding grain antifungal activity and germination energy analysis.

Wheat grains contaminated with DON grown and harvested in 2011 (breeding and seed company *Florimond Desprez*, Cappelle-en-Pévèle, France) were used for LAB bioproducts for the research of grain detoxification.

Barley seeds of malting barley cultivar “Propino” (obtained from the Institute of Agriculture, Kėdainiai, Lithuania) and naturally contaminated spring wheat with *Fusarium* spp. (harvested in 2011, breeding and seed company *Florimond Desprez*, Cappelle-en-Pévèle, France) were used for proteomic analysis.

Samples of Lithuanian barley grain (harvested in 2010, obtained from different farms located in Lithuania) were used for bioethanol production analysis.

The totality of seven analyzed wheat grain series of “Arktis” (LEU 60210) spring wheat cultivar (harvested in 2012, protein content 11–11.5 % was randomly collected in 2014 from several farms in Lithuania, Plungė District Municipality) and used for ozonation analysis.

Before the experiment, all the grains were passed through two sieves (3.5 and 1.0 mm slotted perforations) in order to remove impurities.

**Microorganisms.** *L. sakei* (Ls), *P. acidilactici* (Pa7), and *P. pentosaceus* (Pp8, Pp9 and Pp10) strains, previously isolated from spontaneous Lithuanian rye sourdoughs (KTU, Department of Food Science and Technology) as well as *L. bulgaricus* (Lb) obtained from KTU Food Research Institute (Lithuania) were used for the treatment of barley biomass and permeate fermentation. The bacteria

were cultured at the optimal temperatures for each strain (30°C (Ls); 32°C (Pa7 acidilactici); 35°C (Pp8, Pp9, Pp10), and 42°C (Lb)) in MRS broth prior to their use.

The yeasts *K. marxianus* and *Kluyveromyces marxianus var. bulgaricus* (Biodestruction research laboratory, Institute of Botany, Lithuania) were used for the fermentation of barley biomass. The yeast of *S. cerevisiae* (*Lesaffre Polska* S.A., Poland) was used for the comparison of the fermentation efficiency.

Commercial xylanase Ecopulp TX-200A (AB *Enzymes*, Finland) and amylase StargenTM 002 (*DuPont Industrial Biosciences*, Denmark) preparations were used as a biocatalyst in order to increase the efficiency of the enzymatic degradation of contaminated barley granular starch substrate and non-starch polysaccharides to glucose.

**Media for bioproduct fermentation and antifungal test.** Cheese whey permeate was obtained from JSC Rokiškio pienas (Rokiskis, Lithuania) with the following characteristics (g/100 g): dry residue 6.3 ± 0.1, protein content 0.17 ± 0.05, and total sugar (lactose plus galactose) content 5.67 ± 0.12. The permeate was heated for 20 min. at 90 °C temperature for protein precipitation and sterilized at 121°C temperature for 15 minutes.

Various substances for the biotreatment of grain and the evaluation of their antifungal activity were prepared by using fermentation (24 h) at optimal temperature with tested LAB, as well as with *L. bulgaricus* (Lb) widely used in the dairy fermentation industry. Sterilized distilled water was used for the control treatment of wheat grains.

## 2.2. Methods

**Determination of grain chemical composition and qualitative characteristics.** *The required humidity* was set by using the drying method according to the ratio of the grain weight loss while drying (130 ± 3 ºC temperature) and the constant weight (AACC method 44-15 (2000)).

*The bulk density* was determined by weighing a measured volume and the weight of the container filled with a sample of grains, as well as with the weight of 1000 grains.

*The total nitrogen amount* was determined by the micro-Kjeldahl method according to ICC standard method no. 105/2: 1994. Crude protein content was estimated by using the conversion factor of 5.7 for wheat.

*The starch content* was determined by employing the enzymatic analytical method using Megazyme K-TSTA 05/06 reagents (α-amylase and amyloglucosidase) kit (Megazyme International Ireland Ltd., Ireland).

*The total sugar (lactose) content* was determined by using the enzymatic analysis method and employing Megazyme K-LACGAR 03/14 reagent kit (*Megazyme International Ireland* Ltd., Ireland) supported by hydrolyzing the lactose (pH 5.0) to D-galactose and D-glucose while using β-galactosidase

isolated from *Aspergillus niger*.

*The amount of the soluble dry matter* (SDM) was determined according to AACC method 68-62.

*The total reduction of sugars* was determined colorimetrically by using a dinitrosalicylic (DNS) acid reagent.

*The concentration of ethanol* was determined by using direct distillation and pycnometry.

*The pH* of the fermentation medium was measured by using a pH electrode (PP-15,*Sartorius*, Goettingen, Germany).

*Acidity analysis* was performed by titration with 0.1 N NaOH. One degree (1◦) of acidity corresponds to 1 mL of 1 N NaOH required to neutralize the acids present in 20 mL of filtrate.

*The percentage fermentation efficiency* of the enzyme and yeast strains was calculated on the basis of the theorethical yield based on the relationship between the consumed sugar and alcohol

*The grain microstructure* was evaluated with a scanning electronic microscope (SEM). The infected and healthy grain cross-sectional images were taken by using a scanning electron microscope (SEM) EVO 50 (*LEO Electron Microscopy* Ltd., Cambridge, UK) using an SE (second electron) detector.

**Enzymatic analytical tests.** Whole grain flour (0.5 g) was extracted with 3 ml of particular buffer. The extracts were centrifuged at 10,000×*g* for 10 minutes at 20 $^{o}$C and used for enzyme activity measurements.

*α-Amylase activity* was determined by using ICC Standard method No. 108 (ICC, 1998).

*Endoxylanase activity* was determined with the dinitrosalicylic acid assay (Miller, 1959).

*Protease activity* was determined with Sigma's enzymatic assay of protease by using tyrosine as a standard (Sigma Quality Control Test SSCASE01.001, 1999).

**Acoustic technique.** The wheat grain samples were screened by using a recently-developed portable acoustic spectrometer with penetration. The spectrometer measures in relative units the amplitude of the acoustic signal (Ap) which penetrates the grain matrix over the frequency range of 10–80 kHz. The 15–40 kHz interval was selected as the optimum frequency range. The duration of each measurement was ~10 s. The test was carried out by placing the test portion of 200 g grains into a plastic vessel whose base was covered with a sound-transmitting material. The thickness of the grain layer was 50 mm. The impact of DON contamination on the bulk grain density and the amplitude of the penetrating acoustic signal (Ap) were also investigated by using measurement vessels with different diameters (small: 40 mm, medium: 80 mm and large: 100 mm).

**Determination of mycotoxins.** An ELISA was used to determine the

DON concentrations in contaminated barley and wheat test samples. For this purpose, a three test kit RIDASCREEN®FAST DON (*R-Biopharm* AG, Germany) was used. The ground test portion size used in ELISA was 100 g. The mycotoxin extraction and testing was carried out strictly according to the manufacturer's instructions.

**$CO_2$ concentration analysis.** The $CO_2$ concentration of the storaged wheat grains was determined in plastic-sealed jars packed into a constant (25 ºC) climate chamber (*Sheldon Manufacturing*, US). The cells were maintained at a fixed temperature (25 ± 1°C) and constant relative humidity (60–65 %). The $CO_2$ concentration was measured every 1 h by employing a $CO_2$ sensor (*SenseAir* firm $CO_2$ Engine K30 FR) within 48 hours throughout the sample. The sensor is based on the principle of infrared absorption. The analyzed data was recorded by $CO_2$ Meter software (*GasLab*, US).

**Determination of organic acids.** Lactic acid was determined by using enzymatic test K-DLATE 12/12 ("*Megazyme International Ireland* Ltd.", Ireland). The presence of acetic acid was determined by distilling the investigated samples using while using a Behr S4 Distillation unit (Lab Unlimited UK, *Behr*, Frimley,UK).

**Biotreatment of wheat grains** was performed by mixing an individual bacterial culture suspension with a grain sample (antifungal activity: 10 ml suspension for 60 seeds; grain detoxification: 10 ml suspension for 20 g seeds; germination analysis: 10 ml for 100 seeds) and thoroughly shaken for 30 min. at room (18 ºC) temperature. A control sample was prepared by mixing wheat grain with tap water.

For the antifungal test, the Petri dishes with the stagnant agar medium were uncovered one at a time near an open flame, and 10 grains were placed on the medium in 3 rows (3 grains per row, 4 grains per row, 3 grains per row) by using sterilized tweezers. The closed dishes were put into bags, sealed and transferred to a thermostat maintained at a temperature of 27 °C for 7 days. After 7 days, the antifungal activity was evaluated as a percentage of unmolded grains.

**Ethanol production.** A low-temperature technological process was used for ethanol production under laboratory conditions. For control processing, barley wholemeal (300 g) was mixed with water pre-heated to 90 ºC by using a ratio of 1:6 and kept at the same temperature for 30 min. to in order to reach partial degradation of polysaccharides and decrease microbial activity. A simultaneous liquefaction and saccharification procedure was performed for 90 min. at a temperature of 56–57 ºC by adding StargenTM 002 at a dose of 0.456 AV/ kg (according to recommendations of the manufacturer) and appropriate amounts (1150, 2300, 3450, 4600 and 5750 XU 100 g/grain) of Ecopulp TX-200A. The pH was adjusted with lactic acid to obtain pH = 5.0. The fermentation was carried out at 32 ºC temperature for 48 h in 500 ml glass flasks by adding the inoculum (10 g/l) of appropriate yeast. The whole stillage was dried in an

oven at 50 ºC with aeration for 24 h and used as DDGS for DON analysis.

**Ozone treatment of malting grains.** Initially, 200 g of naturally contaminated wheat seeds were evenly spread over metal mesh in a 40 l plastic box. The generated by OZ-3G generator (Kai Yuan, Guangzhou, China) $O_3$ gas (purity ≥ 99.8 %, "*AGA*", Latvia) at the maximum flow was introduced into the bottom of the box via a plastic tube until the concentration of $O_3$ reached its maximum of 20 mg/l. The concentration was maintained during the ozonation process by regulating the proportion of $O_3$ output from the generator controlled by a portable sensor (A-22 Ozone Sensor, California, USA). The procedure was continued for the remaining seeds which were ozonated for additional 40 and 90 min. at 20 mg/l in order to reach the ozonation duration of 80 min. and 130 min., respectively. All the experiments were performed at room temperature and 65–75 % relative humidity. Three parallel replicates of each sample were obtained.

**Germination analysis.** The seeds were germinated between layers of moist filter paper strips (20 cm × 100 cm). One hundred seeds per roll were placed in rows at regular 5–6 cm intervals from the top edge, leaving 3–4 cm gaps on the sides, a strip of filter paper and loosely rolled. Four rolls per treatment were prepared. The rolls were placed in glass beakers with distilled water (covering the bottom 3 cm of rolls) and incubated at room temperature (18°C) for 3 days. The germination percentages (%) were used to measure the effect of LAB on the sprouting energy of seedlings.

**Proteomic analysis of grain.** Proteins were exctracted from each sample and quantified by the 2-D Quant Kit (*GE Healthcare*, UK) with bovine serum albumin (BSA) as standard.

Electrophoretic analysis of four proteins was carried out on 12 % polyacrylamide gels under reducing conditions by using 1.5 mm thick perspex spacers and glass plates. Electrophoresis was performed with SDS running buffer at 180 V for 1 h until the electrophoretic front was approximately 1 cm from the bottom. The gel was stained with Coomassie brilliant blue R-250. Destaining was performed by soaking the stained gels five times with deionized water.

**Volatile compound quantification by gas chromatography (GC).** Analysis of higher alcohols and methanol was performed by using gas chromatography (GC). A Hewlett Packard 5890 gas chromatograph equipped with a split-splitless injector and a FID detector was used in all measurements. The injection temperature was 200 ºC and helium served as a carrier gas with a flow rate of 1.2 mL $min^{-1}$. Chromatographic separation was accomplished with a Zebron ZB-WAX column (30 m × 0.25 mm × 0.25 µm, 100 % polyethylene glycol; *Phenomenex*, Torrance, CA, USA). The temperature programme was as follows: the initial temperature of 40 ºC for 5 min, rising to 100 ºC at a rate of 4 ºC $min^{-1}$, and a 2 min hold. The detection temperature was set at 250 ºC.

**Mycotoxin determination by UHPLC-MS**. The optimized chromatography conditions were kept constant for both applied instrumental

UHPLC-MS methods. The UHPLC separation of target compounds was carried out by using a 100 mm × 2.1 mm i.d., 2.6 µm Kinetex C18 reversed phase analytical column (*Phenomenex*, USA) operated at 40 °C, by applying a flow rate of 300 µL/min with a mobile phase gradient based on 0.1 % formic acid in water (A) and 100 % methanol (B). The effective gradient began at the initial mobile phase of 95 % A and 5 % B, which was maintained for 6.0 min.; from 6 min. to 10 min., the percentage of phase B was linearly raised up to 95 % and was held constant until 11 min. Then the percentage of phase B was sharply decreased again to 5 % over 0.1 min., and was kept at this level until 15 min. The injection volume of 10 µL was used, and the column and sample temperatures were 40 °C and 10 °C, respectively.

UHPLC-Orbitrap-HRMS analyses were performed on an Accela 1250 UHPLC system coupled to a Q-Exactive Orbitrap-HRMS (*Thermo Fisher Scientific*, US) detection system (Bremen, Germany).

UHPLC-QqQ-MS/MS analyses were performed by using an AB Sciex QTrap 5500 mass spectrometer (AB *SCIEX*, Framingham, MA,US) equipped with heated electrospray ionisation interface and a Waters Acquity UHPLC system (*Waters*, Milford, MA, US).

## 3. RESULTS AND DISCUSSION

### 3.1. The changes in the microstructure and chemical composition of *Fusarium* spp. damaged barley and wheat grains

#### The changes in the chemical composition of *Fusarium* damaged grains

*Fusarium* spp. could considerably influence the structure as well as the chemical composition of barley and wheat grains. Therefore, some components, such as total protein, starch and hectoliter/1000 grain mass were determined.

The relationship between starch, hectoliter/1000 grain mass and DON concentration in barley and wheat grains was found. With a concentration of DON (0–425 µg/kg) in barley grain, starch and barley grain hectoliters content decrease ($r$ = –0.310 and $r$ = –0.328; $p \leq 0.05$). A similar trend was found in wheat grains. With a concentration of DON (254–1600 µg/kg) in wheat grain, the starch content ($r$ = -0.737; $p \leq 0.01$) and 1000 wheat grain weight ($r$ = –0.637; $p \leq 0.01$) significantly decreased.

#### The changes in enzymes activity of *Fusarium* spp. damaged grains

The chemical changes in the endosperm of *Fusarium* spp. damaged kernels caused probably by the activity of hydrolytic enzymes produced by the fungi showing amylolytic, xylanolytic and proteolytic activities on selected barley and wheat samples were analyzed. The results show that the higher contamination level resulted in an increased activity of α-amylase, xylanase and protease.

**Table 3.1.** DON concentration and enzyme activities in naturally *Fusarium* infected barley grains

| Sample No. | DON concentration (µg/kg) | Amylase activity (AU/g) | Xylanase activity (XU/g) | Protease activity (PU/g) |
|---|---|---|---|---|
| 1 | 0 ± 5 | 542 ± 1 | 0.173 ± 0.00 | 86 ± 4 |
| 2 | 10 ± 5 | 560 ± 4 | 0.172 ± 0.01 | 89 ± 6 |
| 3 | 19 ± 8 | 608 ± 3 | 0.173 ± 0.01 | 92 ± 7 |
| 4 | 20 ± 5 | 579 ± 4 | 0.175 ± 0.01 | 99 ± 9 |
| 5 | 28 ± 6 | 568 ± 5 | 0.173 ± 0.01 | 90 ± 2 |
| 6 | 30 ± 4 | 528 ± 9 | 0.173 ± 0.00 | 88 ± 2 |
| 7 | 40 ± 7 | 570 ± 9 | 0.173 ± 0.01 | 92 ± 11 |
| 8 | 55 ± 5 | 586 ± 3 | 0.178 ± 0.01 | 97 ± 1 |
| 9 | 60 ± 8 | 557 ± 4 | 0.173 ± 0.01 | 87 ± 4 |
| 10 | 75 ± 6 | 727 ± 2 | 0.175 ± 0.01 | 107 ± 10 |
| 11 | 90 ± 8 | 721 ± 6 | 0.180 ± 0.00 | 133 ± 8 |
| 12 | 140 ± 8 | 605 ± 4 | 0.177 ± 0.00 | 129 ± 9 |
| 13 | 170 ± 15 | 807 ± 4 | 0.179 ± 0.01 | 159 ± 4 |
| 14 | 425 ± 10 | 913 ± 2 | 0.186 ± 0.01 | 189 ± 7 |

At the starting point of barley contamination (DON concentration till 140 µg/kg), the measured α-amylase activity varied between 528–727 AV/g, xylanase activity between 0.172–0.180 AU/g and protease activity between 86–133 PU/g barley grain (Table 3.1).

By increasing the DON concentration till 425 µg/kg (EU Regulation limit 1250 µg/kg), the enzyme activities increased significantly till 605–913 AV/g, 0.177–0.186 AU/g and 129–189 PU/g, respectively. In all the cases close positive relationships between the enzyme activities and the degree of *Fusarium* infection (DON concentration) were found (for α-amylase, $r = 0.868$; $p \leq 0.01$; xylanase $r = 0.482$; $p \leq 0.01$, and for protease, $r = 0.914$; $p \leq 0.01$).

Similar results were obtained from the analyzed contaminated wheat samples. At the starting point of wheat contamination (DON concentration till 1246 µg/kg), the measured α-amylase activity varied between 229–296 AV/g, xylanase 0.153–0.167 activity between 0.172–0.180 AU/g, and protease activity between 21–213 PU/g wheat grain (Table 3.2).

By increasing the DON concentration till 4371 µg/kg, the enzyme activities increased significantly till 306–464 AV/g, 0.163–0.189 AU/g and 137–397 PU/g, respectively. In all the cases, close positive relationships between the enzyme activities and the degree of *Fusarium* infection (DON concentration) were found (for α-amylase, $r = 0.741$; $p \leq 0.01$; xylanase $r = 0.602$; $p \leq 0.01$, and for protease $r = 0.800$; $p \leq 0.01$).

**Table 3.2.** DON concentration and enzyme activities in naturally *Fusarium* infected wheat grains

| Sample No. | DON concentration (μg/kg) | Amylase activity (AU/g) | Xylanase activity (XU/g) | Protease activity (PU/g) |
|---|---|---|---|---|
| 1 | 0 ± 0 | 229 ± 8 | 0.157 ± 0.00 | 22 ± 4 |
| 2 | 0 ± 0 | 283 ± 9 | 0.167 ± 0.00 | 21 ± 5 |
| 3 | 577 ± 82 | 335 ± 6 | 0.157 ± 0.01 | 152 ± 10 |
| 4 | 892 ± 64 | 296 ± 9 | 0.153 ± 0.00 | 87 ± 8 |
| 5 | 1246 ± 145 | 255 ± 4 | 0.160 ± 0.01 | 213 ± 25 |
| 6 | 1457 ± 106 | 306 ± 19 | 0.189 ± 0.01 | 137 ± 19 |
| 7 | 2750 ± 173 | 321 ± 12 | 0.163 ± 0.01 | 239 ± 17 |
| 9 | 3534 ± 190 | 328 ± 9 | 0.179 ± 0.00 | 173 ± 8 |
| 9 | 3942 ± 278 | 464 ± 13 | 0.185 ± 0.01 | 397 ± 20 |
| 10 | 4371 ± 223 | 365 ± 6 | 0.179 ± 0.01 | 234 ± 17 |

**The changes in the microstructure composition and germination energy of *Fusarium* damaged grains**

Fungal infection not only results in the accumulation of mycotoxins, but also causes grains to shrivel and become more porous. This is known as head blight or scab, one of the indicators of poor grain quality. The microscopic analysis of the contaminated grains by *Fusarium* and the wholesome barley and wheat grains shows visible damage on the surface of the contaminated grain kernels (Fig. 3.1, *1b*). It shows what happens to the structure of the grain kernels when it has been attacked by *Fusarium*.

In Figure 3.1. (*2a*), the structure of the grain kernel wall is healthy and wholesome, in Figure 3.1. (*2b*), the starch granules have been 'consumed' by the fungus and the skeleton type of landscape appears more prominently. Endosperm cells of healthy kernels (Fig. 3.1, *3a*, *4a*) were filled with tightly packed large and small starch granules surrounded by the protein matrix. Examination of the endosperm in the *Fusarium* damaged kernel revealed the presence of hyphen in the endosperm and characteristic micro structural changes observed as lack of cell walls and disappearance of the protein matrix between starch granules and visible symptoms of the amylolitic degradation (and absence) of starch granules (Fig. 3.1, *3b*,*4b*).

These changes in the structure of the damaged grain endosperm influence the porosity of the kernels and change the packing factor of grains in the grain matrix.

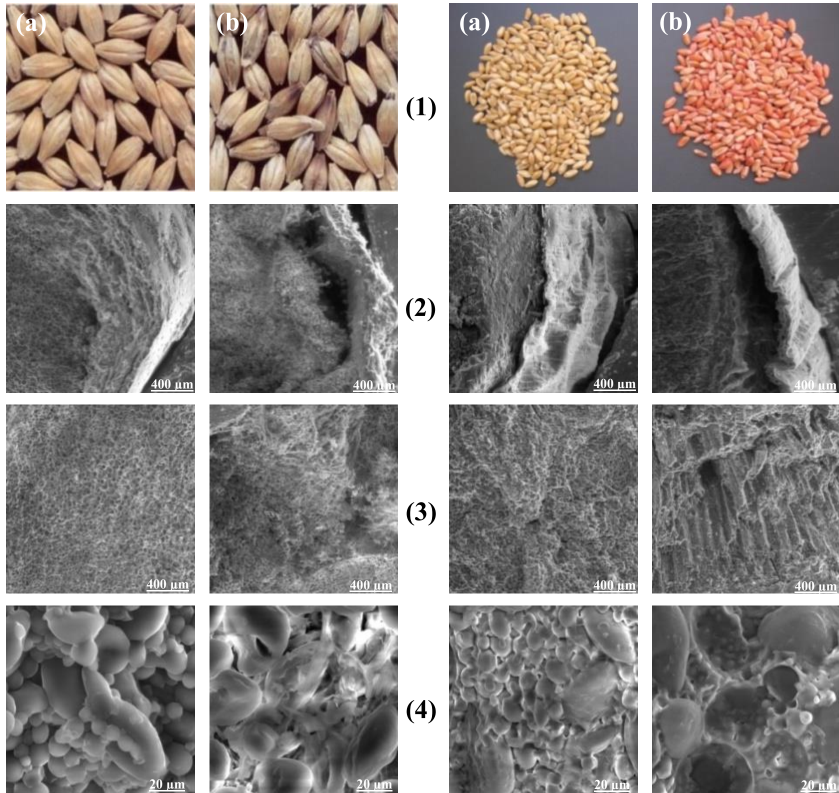



**Fig. 3.1.** The microscopic analysis of the wholesome barley/wheat (*a*) and contaminated grains (*b*) by *Fusarium.* 1 – total grain image, 2 – microstructure of grain wall (400 µm), 3 – endosperm microstructure of grain (400 µm), 4 – starch microstructure of grain (20 µm)

The chemical changes of *Fusarium* damaged wheat kernels caused grain germination energy changes by the fungi contamination. Results show that the higher contamination (DON) level resulted in a decreased wheat grain germination energy (Fig. 3.2). At the starting point of wheat contamination (DON concentration till 1246 µg/kg), the measured germination energy of wheat grains varied between 91.00–99.67 % (Fig. 3.2). By increasing the DON concentration till 4371 µg/kg, the germination energy decreased significantly till 37.00 %

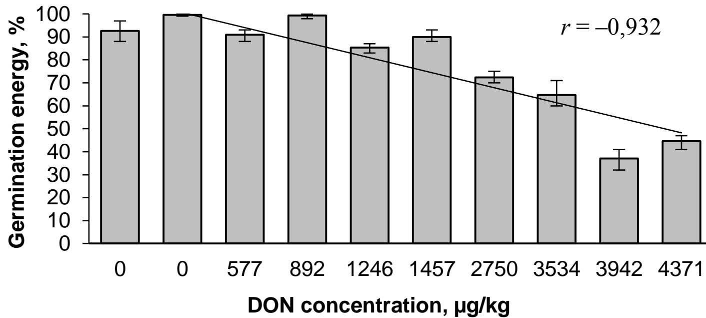



**Fig. 3.2.** The effect of DON concentration on wheat grain germination energy after 3 days of germination

A reverse positive relationship between DON concentration and wheat grain germination was found ($r = -0.932$; $p \leq 0.01$) (Fig. 3.2).

## 3.2. New electrical acoustic technical application research opportunities for DON detection in wheat grains

### Calibration of the acoustic method for DON analysis in wheat grains

Mathematical analysis showed that the analytical curves prepared from different measurements carried out by two different analysts over 2 days were reproducible and stable.

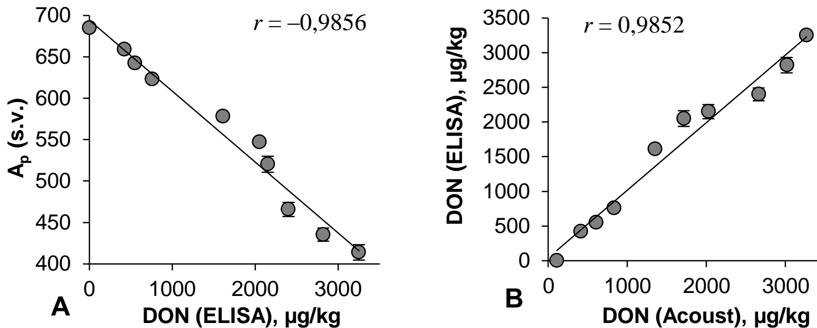



**Fig. 3.3.** Relationship between deoxynivalenol (DON) concentrations (determined by ELISA) in wheat model samples and (*A*) the penetrating acoustic signal amplitude ($A_p$) in relative units (r.u.) or (*B*) the acoustic calibration curve (DON(Acoust) ($n$ =10), where $r$ = coefficient of correlation

A strong inverse correlation was observed between the DON content in the model wheat samples measured by ELISA (DON(ELISA)) and the Ap values recorded by using the acoustic spectrometer ($r = -0.9856$; $p < 0.05$) as shown in Figure 3.3. (*A*). Regression slopes and y-axis intercepts differed significantly

from zero ($p$ < 0.05). For acoustic calibration (Figure 3.3, *B*), the DON concentrations (DON(Acoust)) from each of the model samples were calculated according to the equation $y$ = -0.086x + 694.08 derived from the relationship between DON(ELISA) and $A_p$ (Figure 3.3, *A*). Single laboratory validation based on accepted standards revealed the precise performance characteristics of the acoustic measurements in terms of within-laboratory reproducibility.

The naturally-contaminated soft wheat samples (the validation sample set) were analyzed in order to confirm the relationship between the acoustic signal amplitude and the DON content. A significant linear correlation ($r$ = 0.9013; $p$ < 0.05) between the concentration of DON determined by both ELISA and acoustic method (Fig 3.4) was observed.

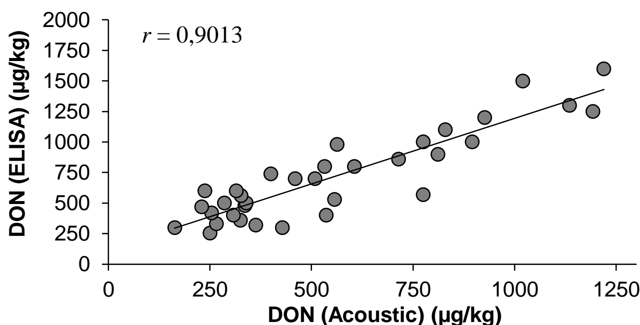



**Fig. 3.4.** Relationship between the deoxynivalenol (DON) concentration determined by ELISA (DON(ELISA)) and the DON concentration calculated from the acoustic calibration curve (DON(Acoust)) in naturally-contaminated soft wheat samples (validation sample set) ($n$ = 34), where $r$ = coefficient of correlation

The relative standard deviation (RSD) values for the wheat model samples varied between 2.1 and 9.3 %, which falls well within the range of repeatability (≤ 20 %) prescribed by CEN/TR 16059:2010 (CEN, 2010). According to our results, the acoustic method is reliable and can be used to predict the level of DON contamination in grain samples.

### 3.3. Distribution of *Fusarium* mycotoxins in malting wheat grains after ozone treatment

The results obtained during the contamination studies are summarized in the box and whisker plots in Figure 3.5. The dependence of the results presented in the graphs indicates a rather different distribution of the initial concentration levels of mycotoxin contamination in the analyzed wheat grain samples, as well as variations of their concentrations at different ozonation stages. The results indicate notable differences in the contamination levels and the degradation range of the analyzed mycotoxins in wheat grain samples. The plots in the graphs

indicate the median values (at 50 % level). The whiskers indicate the extreme (maximum and minimum) concentration levels for each contaminant in grain samples at different stages of ozonation. However, the skewness of the median and the shift of box-plot characters within the concentration range show a positive influence of ozonation treatment on the degradation rate of mycotoxins in the samples, depending on the mycotoxin type and their initial contamination level in wheat grains. Due to the high impact of ozonation on the destruction of mycotoxins, a notable shift of median values to lower concentrations was found for ozonated ZEA and T-2 toxins after treatment for up to 80 min, whereas the trend in the case of DON was less pronounced (Fig. 3.5, *a*).

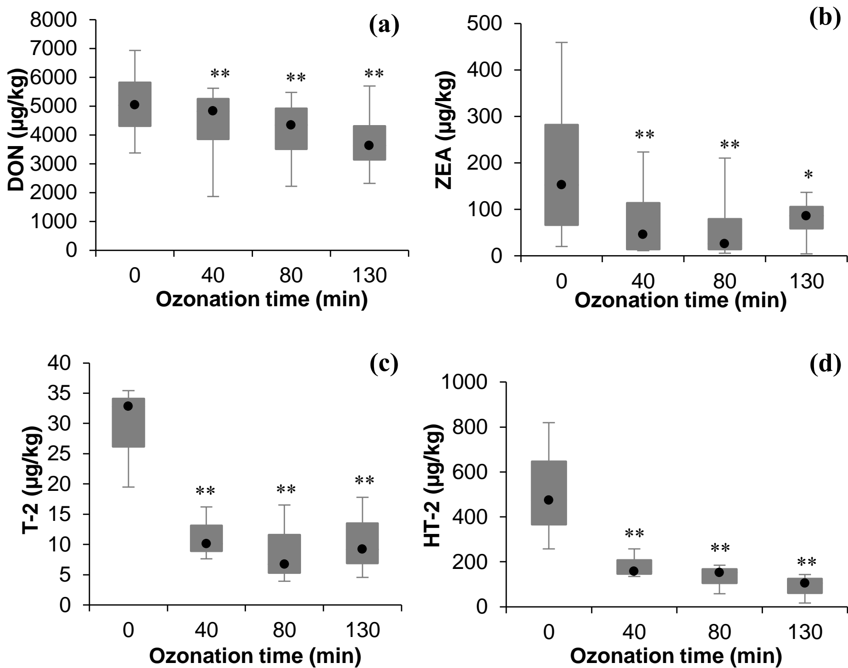



**Fig. 3.5.** The ranges of mycotoxin concentrations (the median values marked with dots) in wheat grain samples vs. the ozonation time: DON (*a*), ZEA (*b*), T-2 (*c*), HT-2 toxin (*d*) (* and ** – substantial differences compared with the control at 95 and 99 % probability level)

The presence of DON and ZEA was found in four of the analyzed five malting wheat samples. The initial levels of DON varied from 3370 µg/kg to 6930 µg/kg with the average concentration of 5093 µg/kg (Figure 3.5, *a*). The levels of ZEA were in the range from 19.5 µg/kg to 459 µg/kg, with the average concentration of 196 µg/kg (Figure 3.5, *b*). The amounts of T-2 and HT-2 toxins

detected in three samples (in one sample as the only contaminant and in two samples together with ZEA and DON) initially varied from 19.54 μg/kg to 35.4 μg/kg (Figure 3.5, *c*) and from 258.4 μg/kg to 819 μg/kg (Figure 3.5, *d*), respectively. The average concentrations determined for T-2 and HT-2 were 32.8 μg/kg and 517 μg/kg, respectively.

The extent of mycotoxin degradation increased with the processing time. The influence of ozonation was less obvious in the case of DON, as its concentration was reduced on average by only 16 % after the first 40 min. of exposure to 20 mg/l of $O_3$ and reached the mean concentration of 4283 μg/kg. The maximum degradation efficiency of 25 % after 130 min. of ozonation allowed to reduce the contamination by 1.3 times; meanwhile, the average contamination level remained above the acceptable level of 1250 μg/kg (EC, 2007). For other three *Fusarium* mycotoxins, a significant decrease in concentration was notable already after the first 40 min. of treatment with 20 mg/l of $O_3$, resulting on average in 2.4-fold, 2.8-fold, and 2.6-fold reduction of contamination with ZEA, HT-2, and T-2 mycotoxins, respectively. It could be concluded that, for these mycotoxins, even a short ozone treatment was highly effective, resulting in 58.6 %, 65.6 %, and 62.0 % degradation, while also taking into account the rather low initial concentrations of natural contamination levels in the analyzed wheat samples, compared to those of DON.

On average, the maximum degradation rates of DON, ZEA, HT-2, and T-2 toxins after ozonation for 130 min. were 25.0 %, 48.7 %, 82.8 %, and 68.9 %, respectively. It can thus be concluded that $O_3$ treatment provides effective means for reducing DON, ZEA, T-2, and HT-2 toxin levels in contaminated malting wheat grains, and has a potential for ensuring that DON levels in stored malting wheat grains, as well as ZEA and other trichothecenes, could be kept below the maximum acceptable levels in accordance with EU legislation (EC 2007, 2013).

A second order (e.g., *parabolic*) polynomial fitting was the most suitable method for indicating the relationships between the duration of ozone treatment and the degradation extent of DON, ZEA, HT-2, and T-2 toxins, as shown in Figure 3.6. The fitted parabolic function curves indicate the determined correlations between the time ($x$) and the degradation extent ($y$). The fit was found to be acceptable for all mycotoxins, as proved by the correlation coefficient ($R^2$) values of 0.9721, 0.9087, 0.9644, and 0.9620 for the curves of DON, ZEA, T-2, and HT-2. In our study, all *Fusarium* mycotoxins were partially degraded as they were exposed to $O_3$ treatment. However, ozonation resulted in different degradation rates for the tested trichothecenes.

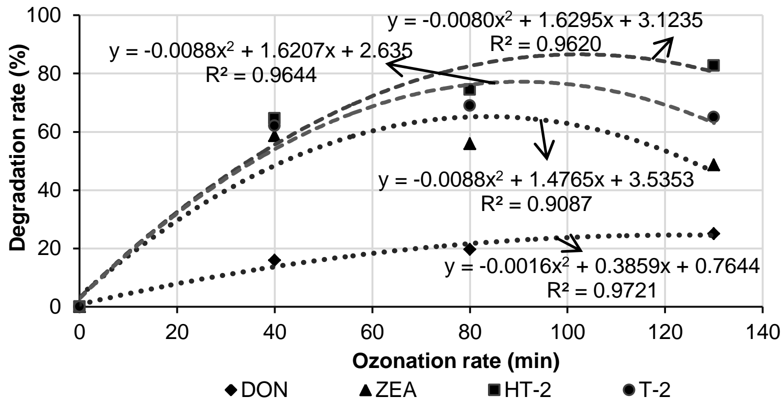



**Fig. 3.6.** The effect of exposure time to 20 mg/l of ozone on the destruction of mycotoxins in malting wheat

In some cases of highly contaminated malting wheat, the level of DON could be reduced by up to 25 % after $O_3$ treatment at the concentration of 20 mg/l for 130 min., while in other cases the amount of ZEA was reduced by up to 55 % after ozonation for 80 min. Similarly, the HT-2 and T-2 toxins were most effectively degraded up to 74.5 % and 70.0 % after treatment with 20 mg/l of ozone for 80 min.

## 3.5. Antifungal LAB bioproduct for detoxification and increasing efficiency of fermentation process from *Fusarium* spp. contaminated grains

### The effect of LAB metabolites on the mycotoxin content in wheat grains

The results obtained during the contamination studies are summarized in the box and whisker plots in Figure 3.7.

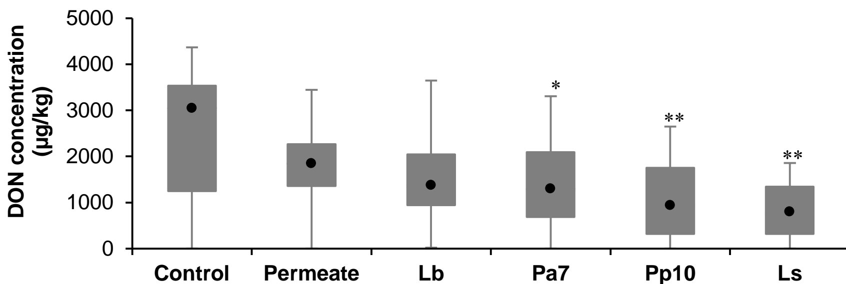



**Fig. 3.7.** The effects of treatment with LAB bioproducts on the DON concentration in contaminated wheat samples (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

The presence of DON was found in three of the analyzed four malting wheat samples. The initial levels of DON varied from 1246 µg/kg to 4371 µg/kg with the average concentration of 3050 µg/kg (Figure 3.7). The ELISA analysis showed that the DON content in contaminated wheat grains after treatment by LAB bio products fermented in permeate medium decreased. Detoxification level depended on the LAB strain used in grain biotreatment. The study showed that the DON concentration in wheat grains after processing with LAB bio products led to a reduction of DON concentration from 16.59 to 66.86 % compared to the control values (Fig. 3.7).

The highest detoxification effect established after the treatment of contaminated wheat grain with *L. sakei* (Ls) (from 57.47 to 66.86 %; $p \leq 0.01$) and *P. pentosaceus* (Pp10) (from 39.46 to 66.21 %; $p \leq 0.01$) compared to the control values. Other types of LAB influence of the concentration of DON in wheat grain was lower with *P. acidilactici* (Pa7) (from 24.30 to 52.35 %; $p \leq 0.05$) and *L. bulgaricus* (Lb) (from 16.59 to 64.74 %). Wheat grain treatment with unfermented permeate also observed DON concentration decrease from 21.14 % to 46.94 % compared to the control values.

**Antifungal trials of bioproducts based on cheese permeate**

Permeate samples fermented by tested LAB were screened for their antifungal activity against various *Fusarium* spp. possibly occurring on infected wheat grains (Fig. 3.8). The inhibitory effect of permeate bioproducts was quite different, mainly because various samples had different amounts of lactic acid present in them.

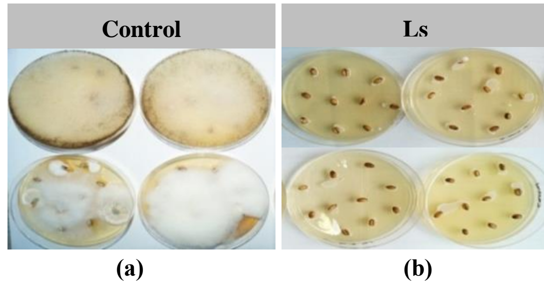
**Table 3.3**. Unmolded grain content after different biotreatments

| Grain sample | Unmoulded grain, % |
|---|---|
| Control[1] | 21.7 ± 1.2 |
| Permeate | 45.0 ± 1.6** |
| Permeate + *L. bulgaricus* (Lb) | 95.0 ± 1.4** |
| Permeate + *L. sakei* (Ls) | 93.3 ± 1.7** |
| Permeate + *P. acidilactici* (Pa7) | 91.5 ± 2.1** |
| Permeate + *P. pentosaceus* (Pp8) | 87.5 ± 2.3** |
| Permeate + *P. pentosaceus* (Pp9) | 88.0 ± 2.0** |
| Permeate + *P. pentosaceus* (Pp10) | 88.3 ± 1.5** |

[1]Control – grain treated with sterilized distilled water.
** – substantial differences compared with the control at 99 % probability level

The antifungal test has shown that the grain treatment with pasteurized cheese whey permeate fermented by LAB significantly ($p < 0.05$) improved the grain resistance to mould infection, with more than 88 % (*P. pentosaceus* strains), and on average 93 % (*L. sakei*, *P. acidilactici* and *L. bulgaricus*) of unmoulded grains compared to the control (Table 3.3). The low antifungal

activity was observed in the samples treated with spontaneously fermented permeate, which resulted in 45 % of unmoulded grains.




**(a)** **(b)**

**Fig 3.8.** *Fusarium* spp. contaminated wheat grain (*a*) and after biotreatment with Ls (fermented in permeate 48 h) (*b*)

The production rate of organic acids during permeate fermentation strongly ($p < 0.05$) depended on the LAB strain and fermentation time.

**Table 3.4.** L(+) ir D(-)-lactic acid amount (g/l) in LAB fermented bioproduct

| Fermentation time, h. | LAB | L(+)-lactic acid, g/l | D(-)-lactic acid, g/l | Total lactic acid, g/l |
|---|---|---|---|---|
| 24 | Lb | 0.356 ± 0.04 | 0.417 ± 0.05 | 0.773 ± 0.12 |
| | Ls | 0.505 ± 0.06 | 0.417 ± 0.05 | 0.922 ± 0.14 |
| | Pa7 | 0.646 ± 0.08 | 0.449 ± 0.05 | 1.095 ± 0.16 |
| | Pp8 | 0.388 ± 0.05 | 0.384 ± 0.05 | 0.772 ± 0.12 |
| | Pp9 | 0.517 ± 0.06 | 1.826 ± 0.22 | 2.343 ± 0.35 |
| | Pp10 | 1.648 ± 0.20 | 0.545 ± 0.07 | 2.193 ± 0.33 |
| 48 | Lb | 0. 000 ± 0.00 | 1.697 ± 0.20 | 1.697 ± 0.20 |
| | Ls | 1.134 ± 0.12 | 1.021 ± 0.11 | 2.155± 0.28 |
| | Pa7 | 0. 000 ± 0.00 | 4.870 ± 0.54 | 4.870 ± 0.54 |
| | Pp8 | 0.632 ± 0.07 | 0.456 ± 0.05 | 1.088 ± 0.14 |
| | Pp9 | 0.527 ± 0.06 | 0.249 ± 0.03 | 0.776 ± 0.10 |
| | Pp10 | 1.034 ± 0.11 | 0.481 ± 0.05 | 1.515 ± 0.20 |
| 72 | Lb | 0.000 ± 0.00 | 1.371 ± 0.19 | 1.371 ± 0.19 |
| | Ls | 0.924 ± 0.13 | 0.633 ± 0.09 | 1.557 ± 0.23 |
| | Pa7 | 0.646 ± 0.09 | 0.000 ± 0.00 | 0.646 ± 0.09 |
| | Pp8 | 0.737 ± 0.10 | 0.233 ± 0.03 | 0.970 ± 0.15 |
| | Pp9 | 0.420 ± 0.06 | 0.000 ± 0.00 | 0.420 ± 0.06 |
| | Pp10 | 0.485 ± 0.07 | 0.449 ± 0.06 | 0.934 ± 0.14 |

Pa7 produced the highest amount of total lactic acid (LA) (4.87 g/l) after 48 h of fermentation; in this case only D(-)-lactic acid has been detected (Table 3.4). The highest total LA content on average 2.26 g/l was produced by Pp10 and Pp9 strains after 24 h of fermentation. Ls and all *P. pentosaceus* (Pp8, Pp9 and Pp10) strains could be indicated as DL-forming bacteria, whereas the percentage

of L(+)-lactic acid in which varied between 52–76 %.

The highest content of acetic acid (AA) after 24 h of fermentation was found in Lb and Ls samples (5.22 and 4.14 g/l, respectively), while AA production by the other LAB strains was lower by 58.4 % (Fig. 3.9), and AA content was only from 12.5 to 17.6 % higher compared to the control values.

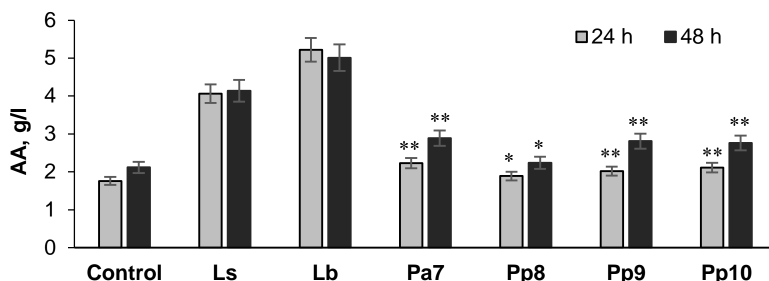



**Fig 3.9.** The acetic acid (AA) content after 24 and 48 hours of cheese permeate fermentation by different LAB strains (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

Prolonged fermentation (48 hours) with Pa7 and Pp10 allowed to increase the content of acetic acid on average by 14.4 %, Pp9 and Pp8 on average by 21.4 %, and Ls by 5.5 % compared to 24 h fermentation. According to the data analysis, a strong relation ($r$ = 0.832; $p \leq 0.05$) was found between the AA content in LAB bioproducts and their antifungal activity. The assessment of the impact on antifungal activity showed that the correlation coefficient between the total lactic acid content in bioproduct samples and the antifungal activity was lower ($r$ = 0.488; $p \leq 0.05$). No significant correlation was found between the content of lactic acid isomers L(+) and D(-) in bioproduct samples and antifungal activity.

### The effect of LAB metabolites on the germination energy in contaminated wheat grains

The effect of biotreatment on germination energy significantly ($p < 0.05$) depends on LAB strains used for the bioproduct fermentation, as well as on the grain contamination level. The germination energy of the contaminated grains (1800 µg/kg DON) decreased on average by 9 % compared with the healthy grains (<10 µg/kg DON) (Fig. 3.10). The Pa7 and Pp10 bioproducts containing the high amounts of LA (Table 3.4) and low amounts of acetic acid (AA) (Fig. 3.9) had no significant effect on the germination energy of healthy grains, whereas the germination energy of contaminated grains increased on average by 7.5 % in comparison with the untreated grains.

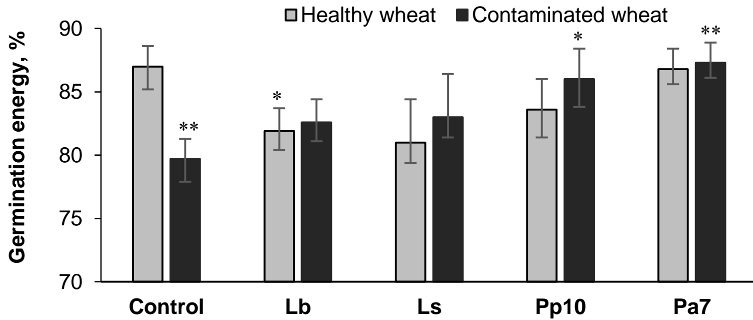



**Fig. 3.10.** The effect of permeate bioproduct fermented with different LAB strains on healthy (0 µg/kg DON) and contaminated (1800 µg/kg DON) wheat grain germination energy after 3 days of germination (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

The Ls and Lb bioproducts containing the high LA and low AA contents lowered the germination energy of healthy grains by 6.9 %. However, the germination energy of contaminated grains was increased by 3.6 % in comparison with the control values.

### Fermentation of *Fusarium* spp. contaminated barley biomass

In order to determine the efficiency of the alcohol fermentation of barley contaminated at different levels with *Fusarium* spp. mycotoxin deoxynivalenol (DON), the concentration of ethanol and fusel oils were analyzed.

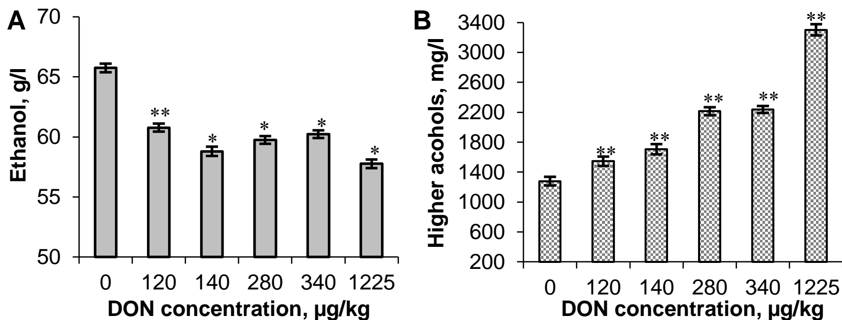



**Fig 3.11.** Effect of contamination with DON on the amount of ethanol (*A*) and concentrations of higher alcohols (*B*) in the distillates from *Fusarium* spp. contaminated barley (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

It was found that liquefaction and saccharification of *Fusarium* contaminated barley biomass using only traditional amylolytic enzymes

(StargenTM 002; 0.456 GU/kg) caused the decrease between 7.5 % (sample B-120) and 12.2 % (sample B-1225) in ethanol concentrations (Fig. 3.11 A). Figure 3.11 B shows on the contrary a different picture for the higher alcohols where an increase between 17.4 % (sample B-120) and 61.3 % (sample B-1225) in concentrations compared to the control sample may be noticed. The decrease of ethanol concentration was not adequate to the changes of higher alcohols. It indicates that apart from mycotoxins, many factors could modify the fermentation process and the composition of raw spirits.

The ethanol yields after fermentation of wholesome and infected barley biomass reached 79 % and 71 % of the theoretical value (0.461 g/g), respectively (Table 3.5). The explanation for the decrease in the ratio of fermentation by *S. cerevisiae* might be the inhibitory effect of high concentrations of *Fusarium* mycotoxins on yeast growth.

**Effects of combined enzymatic treatment of infected barley biomass on ethanol yields and fusel oils**

In order to determine the influence of a combined treatment with a mixture of amylolytic and xylanolytic enzymes on the efficiency of the alcohol fermentation process from *Fusarium* contaminated barley biomass, *T. reesei* xylanase (*Ecopulp*) was selected as a catalyst for further trials.

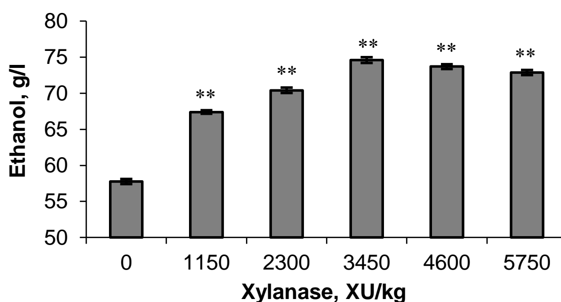



**Fig. 3.12.** Alcohol concentrations in distillate obtained after saccharification of barley (sample B-1225) using appropriate amounts of xylanase (Ecopulp TX-200A) and fermentation with *S. cerevisiae* yeast (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

The addition of a selected catalyst (Ecopulp TX-200A) at the levels from 1150 to 5750 XU/kg in combination with *S. cerevisiae* yeast resulted in a higher on average by 10.3–29.2 % ethanol concentration compared to the control sample without xylanase (57.7 g/l) (Fig. 3.12). The highest ethanol concentration was achieved when the amount of 3450 XU/kg xylanase was added (74.6 g/l). Our study showed that the action of xylanase depends on substrate selectivity. It could be that the hydrolysis rate decreased with the increasing concentration of

soluble materials in the substrate due to the enlarged arabinoxylans degradation and solubilization with the addition of xylanase at the dose higher than 3450 XU/kg.

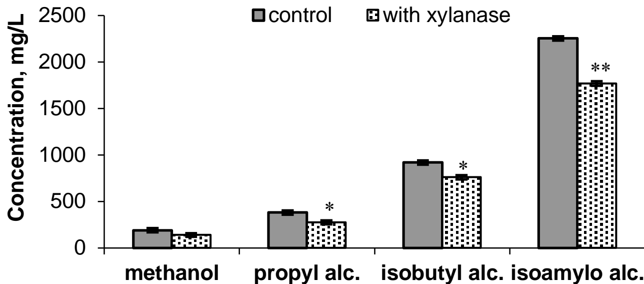



**Fig. 3.13.** Formation of methanol and higher alcohols during fermentation of *Fusarium* contaminated barley (sample B-1225). Samples: control – with amylolytic enzymes; with xylanase – combined treatment with amylolytic enzymes (StargenTM 002) and xylanase (Ecopulp TX-200A) at a level of 3450 XU/kg (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

The quantitative analysis of alcohol showed that by application of a combined enzymatic treatment by addition of *T. reesei* xylanase for hydrolysis of infected barley biomass, also the quality of bioethanol decreasing the total content of higher alcohols (fusel oils) in the distillate on average by 23.9 % improved (Fig. 3.13). It also reduced the methanol concentration by 26.3 % in comparison with the sample without xylanase. In summary, the addition of xylanase played a positive role on the enzymatic hydrolysis of *Fusarium* contaminated barley. The formation of methanol during the fermentation indicated that *Fusarium* contaminated cereals were a pure growing medium for yeasts.

Study of saccharification of wholesome and *Fusarium* spp. contaminated barley biomass (sample B-1225) showed that the treatment with only traditional amylolytic enzymes (0.456 GU/kg) caused the formation of a lower content (by 12.2 % and by 16.3 %, respectively) of reducing sugars in the wort compared to the combined treatments (Table 3.5). The analysis of broth acidity indicated the formation of higher amounts of organic acids during the fermentation of infected barley biomass. The pH value of the broth was lower by 24.2 % in comparison to the uninfected sample (Table 3.5). After a combined enzymatic treatment, the ethanol yields reached 87 % of the theoretical control sample and 83 % of the theoretical infected sample (Table 3.5). The results showed that *T. reesei* xylanase together with different side glycoside hydrolases (cellulase/glucanase) may influence the enrichment of the yeast growing medium.

**Table 3.5.** Effects of added enzymes on reducing sugar (g per 100 g) and ethanol yields following the hydrolysis

| Sample | Worth | | | Fermented broth | | | Ethanol yields* | |
|---|---|---|---|---|---|---|---|---|
| | Reducing sugar | | | Reducing sugar | | | | |
| | Amylolytic enzymes | Amylolytic and xylanase enzymes | pH | Amylolytic enzymes | Amylolytic and xylanase enzymes | pH | Amylolytic enzymes | Amylolytic and xylanase enzymes |
| B-0 | 18.1±0.2[a] | 20.3±0.2[c] | 6.07 | 3.5±0.02[a] | 2.2±0.02[c] | 5.12 | 0.363[a] | 0.403[c] |
| B-1225 | 16.4±0.1[b] | 19.6±0.2[d] | 7.01 | 4.2±0.02[b] | 2.6±0.02[d] | 3.88 | 0.328[b] | 0.381[d] |

**Table 3.6.** Effect of treatment by different LAB on the methanol and higher alcohols of infected barley (sample B-1225) using *S. cerevisiae*, *K. marxianus* and *K. marxianus bulgaricus* yeasts

| Treatment with | Yeast | Methanol | Propyl alcohol | Isobutyl alcohol | Isoamyl alcohol | Total higher alcohols | Ethanol yields[1] |
|---|---|---|---|---|---|---|---|
| Control with amylolytic and xylanase enzymes | *S. cerevisiae* | 140 ± 23 | 275 ± 21 | 760 ± 42 | 1768 ± 174 | 2803 | 0.381 |
| Ls | *S. cerevisiae* | 98 ± 4 | 267 ± 21** | 699 ± 16 | 1957 ± 51 | 2923 | 0.378 |
| | *K. marxianus* | 120 ± 12 | 330 ± 23** | 1132 ± 57** | 2951 ± 48** | 4413 | 0.375 |
| | *K. marxianus bulgaricus* | 118 ± 8 | 415 ± 18** | 680 ± 22* | 2223 ± 28* | 3318 | 0.401 |
| Pa7 | *S. cerevisiae* | 69 ± 3* | 319 ± 32* | 727 ± 24 | 2500 ± 36* | 3546 | 0.375 |
| | *K. marxianus* | 112 ± 6 | 220 ± 22** | 932 ± 14** | 2483 ± 43* | 3635 | 0.373 |
| | *K. marxianus bulgaricus* | 90 ± 4* | 382 ± 24** | 822 ± 11 | 2245 ± 66* | 3449 | 0.392 |
| Pp9 | *S. cerevisiae* | 68 ± 5* | 451 ± 26** | 1080 ± 48** | 2381 ± 38* | 3912 | 0.377 |
| | *K. marxianus* | 92 ± 0 | 330 ± 16** | 926 ± 36** | 3040 ± 42** | 4296 | 0.376 |
| | *K. marxianus bulgaricus* | 88 ± 0 | 540 ± 26** | 618 ± 12* | 2717 ± 21** | 3875 | 0.393 |

[1] Ethanol yields in g alcohol/g reducing sugars

*and ** – substantial differences compared with the control at 95 and 99 % probability level

**Effect of barley grain treatment with LAB on ethanol yields and quality**

Effects of treatment by different LAB on the concentrations of ethanol and higher alcohols in the distillates obtained from *Fusarium* spp. contaminated barley are presented in Figure 3.14 and Table 3.6.

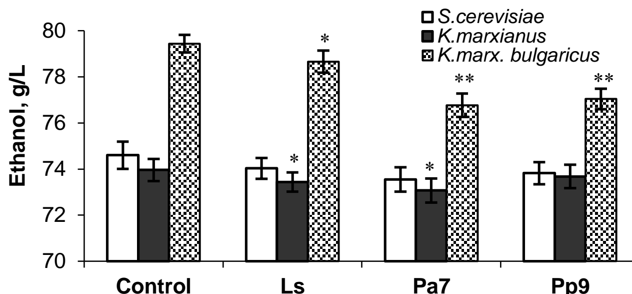



**Fig. 3.14.** Effects of treatment by different LAB of infected barley (sample B-1225) on the ethanol concentrations after fermentation with *S. cerevisiae*, *K. marxianus* and *K. marxianus bulgaricus* yeasts (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

The treatment of contaminated barley with selected LAB and *S. cerevisiae* yeast had no significant influence ($p < 0.05$) on the ethanol quantity (Fig. 3.14). The alcohol concentration after treatment of infected barley with Ls, Pa7 and Pp9 were detected on average of 73.7 g/l. Unfortunately, by using some LAB strains, higher amounts of higher alcohols formed during fermentation (Table 3.6). It was noticed that the application of a combined treatment with xylanase and tested Pa7 and Pp9 for infected barley grain using *S. cerevisiae* yeast for fermentation significantly increased (by 27.5 %) the content of isoamyl alcohol (Table 3.6). Also, the total amount of fusel oils increased by 24.6 % in comparison with the untreated sample (Fig. 3.14). The treatment of barley grains with Ls had a lowering effect on fusel oil formation: the increase in concentrations of isoamyl alcohol by 9.6 % and in total fusel oils by 4.1 %, was observed.

**The selection of yeasts for barley biomass fermentation**

The effects of different yeasts on the amount of ethanol and concentrations of higher alcohols in the distillates from *Fusarium* contaminated barley are presented in Figure 3.14 and Table 3.6. The results showed that the efficiency of the fermentation process of infected barley could be increased by selecting different yeast strains. By using *K. marxianus* var. *bulgaricus* yeast for barley fermentation, higher ethanol concentrations by 5.7 % and 6.5 % (Fig. 3.14) were observed in comparison with *S. cerevisiae* and *K. marxianus* yeast. Treatment with different LAB of barley biomass by using different yeasts for fermentation gave the ethanol yields in the range of 81–86 % of the theoretical value. Based

on the reducing sugar content in barley biomass, after treatment with 20 % of LAB suspension at the xylanase of 3450 XU/kg grain together with 10 g/l of selected yeast, the ethanol yield was obtained (Table 3.6).

*K. marxianus* var. *bulgaricus* yeast in combination with tested LAB gave the highest ethanol yields (85–86 %) of the theoretical value. The results of ethanol qualitative analysis showed that by application of *K. marxianus* yeasts and combined treatment with *T. reesei* xylanase and different LAB, the quality of the obtained bioethanol was lower. After treatment with Ls, Pa7 and Pp9, the higher amounts of total fusel oils in the distillate on average by 26.6 % were identified (Table 3.6) in comparison with the untreated sample (2803 mg/l).They did not differed significantly from the samples fermented with *S. cerevisiae*. Contrary to the treatment with Ls, Pa7 and Pp9 and both *K. marxianus* strains reduced the methanol concentration in the broth by 15 %, 27 % and 35.5 %, respectively, in comparison with the untreated sample (140 mg/l).

### Effect of combined treatments of infected barley on DON contents in DDGS

After alcoholic fermentation using amylolytic enzymes (amyl) for barley biomass hydrolysis, 92 % of the initial DON level (1427 μg/kg d.m.) was detected in DDGS (Fig. 3.15). The complex enzymatic treatment with *T. reesei* xylanase (amyl+xyl) used for wort saccharification reduced the DON levels in DDGS up to 71 % of the initial concentration.

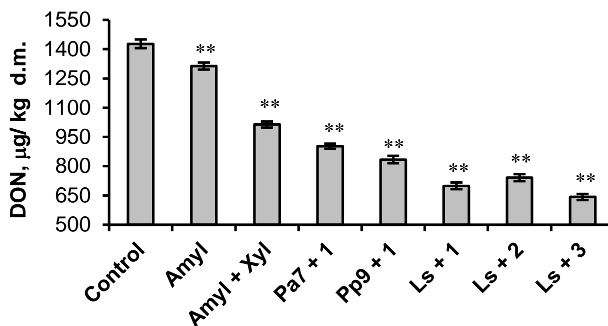



**Fig 3.15.** Effect of different treatments on DON concentrations in DDGS. 1 – *S.cerevisiae*; 2 – *K. marxianus*, 3 – *K. marxianus bulgaricus* (*and ** – substantial differences compared with the control at 95 and 99 % probability level)

However, the residual DON concentration in DDGS was still too high – despite its reduction – for the DDGS to be used in feed. The combined treatment of the infected barley biomass with Ls and *S. cerevisiae* for bioethanol production initiated the reduction of DON levels in DDGS up to 49 % of the initial contamination. Other tested yeasts of *K. marxianus* and *K. marxianus* var. *bulgaricus* in combination with the treatment with Ls reduced the DON concentrations in DDGS up to 52 % and 45 % of the initial level, respectively, during the fermentation of infected barley biomass (Fig. 3.15).

## 4. CONCLUSIONS

1. It was found that *Fusarium* spp. produced metabolites affected cereal chemical composition, microstructure and properties of technological changes:
1.1. With the increasing concentration of DON in barley and wheat grains, the quantity of starch, on the contrary, declined. The infected grain showed an increase in the enzymatic activities (amylolytic, xylanolytic and proteolytic).
1.2. *Fusarium* spp. infection changed the microstructure of grain: the grain wall became thin and wrinkled, it lost the form of starch grains, signs of hydrolysis and increased spacing between the starch granules were determined;
1.3. *Fusarium* spp. contamination had a negative effect on the technological properties of wheat 1000 grain mass and germination energy: the increasing concentration of DON wheat germination energy reduced from 93.6 to 37.00 %.
2. The development of the acoustic method for the detection of mycotoxins in wheat grains showed:
2.1. Strong correlations ($r$ = 0.9013; $p < 0.05$) were achieved between DON concentrations determined by the reference ELISA method and were predicted with the acoustic technique in wheat samples.
2.2. Relative standard deviation (RSD) values for the wheat model samples fall well within the range of repeatability (≤ 20 %) prescribed by CEN/TR 16059:2010 (CEN, 2010). According to our results, the acoustic method is reliable and can be used to predict the level of DON contamination in wheat grain samples.
3. $CO_2$ concentration in stored wheat grains is highly dependent on the degree of *Fusarium* spp. infection in wheat cereal, grain moisture and coarseness:
3.1. It was found that varying moisture content of stored healthy (0 μg/kg) and contaminated (800 μg/kg) wheat grains leads to the increase of $CO_2$ concentration. $CO_2$ concentration increased with the grain moisture content (18.5 and 24.5 %), whereas at 14.5 % a significant concentration increase was not identified.
3.2. The intensified $CO_2$ concentration was determined in large stored grains compared to the small stored grains.
3.3. In *Fusarium* spp. contaminated storaged wheat grains, a higher $CO_2$ concentration than in healthy grains was determined.
4. The largest mycotoxin detoxifying effect of ozonation depended on the duration of the exposure time: when the processing time was 130 minutes,the degradation rate of mycotoxins DON, ZEA, T-2 and HT-2 in

malting grains reached up to 25.0 %, 48.7 %, T-2 65.0 % and 82.8 %, respectively.

5. The development of LAB bio product in malting grain processing provides that:

5.1. The cheese whey permeate is a suitable medium for fermentative production of LAB bio product and its antimicrobial metabolites – lactic and acetic acid.

5.2. The DON concentration in malting wheat reduced at a rate from 16.59 % to 66.86 % after treatment of malting wheat with LAB bio products. A higher antifungal effect was determined with *L. bulgaricus* (Lb) (95 %), *L. sakei* (Ls) (93.3 %) and *P. acidilactici* (Pa7) (91.5 %) strains.

5.3. The treatment with Pa7 and Pp10 fermented permeate increased the germination energy of *Fusarium* spp. contaminated wheat grains on average by 7.5 % compared to the control sample.

6. Proteomic analysis of *Fusarium* spp. contaminated wheat protein substances evaluated by 1D PAGE electrophoresis showed that LAB biotreatment had no significant effect on the malting wheat proteomic changes during grain germination.

7. The examination effect of bio product for the increasing efficiency of bioethanol production from *Fusarium* spp. contaminated barley biomass provides:

7.1. It was found that DON concentration from 120 µg/kg to 1225 µg/kg in *Fusarium* spp. contaminated barley caused the decrease from 7.5 % to 12.2 % in ethanol concentrations and the increase from 21.0 % to 158.43 % in the concentrations of higher alcohols compared to control sample (DON – 0 µg/kg).

7.2. The highest ethanol yield (74.6 g/l) was obtained by using xylanase of 3450 XU/kg grain for hydrolysis; xylanase caused the decrease to 23.9 % in the concentrations of higher alcohols.

7.3. Treatment with Ls of *Fusarium* spp. contaminated barley biomass using *K. marxianus* var. *bulgaricus* yeast for fermentation gave the ethanol yields reaching 87 % of the theoretical value for the control sample.

7.4. The tested yeasts of *K. marxianus* and *K. marxianus* var. *bulgaricus* in combination with the treatment by Ls allowed to reduce the DON concentrations in DDGS up to 45 % of the initial level, respectively, during the fermentation of the infected barley biomass.

## LIST OF PUBLICATIONS

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Bartkienė, E. (2015). Antimicrobial Activities of BLIS Producing Lactic Acid Bacteria in Combination with Acoustic Screening of Mycotoxins to Increase Malting Grains Safety and Quality // In: *Healthy Grain for a Healthy Diet: 20th International Scientific Conference: Book of Abstracts* / Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke (DIfE), 2015. p. [1].

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# *FUSARIUM* SPP. PAŽEISTŲ JAVŲ GRŪDŲ SALYKLO GAMYBOJE APTIKIMAS IR DETOKSIKACIJA ANTIMIKROBINĖMIS BIOPRIEMONĖMIS

## SANTRAUKA

**Temos aktualumas.** Gyventojų skaičius Žemėje nuolat didėja. Tai lemia maisto produktų ir pašarų paklausos augimą. Grūdinė žaliava maisto produktų gamyboje užima svarbią vietą ir sudaro didelę žmogaus mitybinio raciono dalį, todėl labai svarbu užtikrinti, kad ji atitiktų griežtus kokybės ir saugos reikalavimus.

Viena iš didžiausių grūdinės žaliavos panaudojimo sričių yra salyklo gamyba, kuri yra svarbi maisto pramonės šaka. Pastaruoju metu ypač aktualus salyklo gamybos efektyvumo didinimas. Šios problemos sprendimas susijęs su salyklinių grūdų daigumo didinimu įvertinant ir laiku iš grūdų perdirbimo grandinės pašalinant mikroskopinių grybų pažeistus salyklinius grūdus. Nepalankios klimatinės salyklinių grūdų auginimo bei netinkamos jų laikymo sąlygos ir grūdų morfologiniai ypatumai sudaro palankias sąlygas daugintis mikroorganizmams. Todėl ypatingas dėmesys turi būti skiriamas antrinių mikroskopinių grybų metabolizmo produktų – mikotoksinų – prevencijai. Dėl grūdų užteršimo mikotoksinais patiriami didžiuliai ekonominiai nuostoliai, nes užkrėsta žaliava negali būti parduodama ar perdirbama. Taip pat pažymėtina, kad Lietuvoje sparčiai plečiama ir biodegalų gamyba. Žinoma, kad užkrėsta grūdinė žaliava turi neigiamos įtakos fermentacijos procesui, o kaip bioetanolio gamybos atliekos lieka žlaugtai, kurie gali būti sėkmingai naudojami kombinuotųjų pašarų gamyboje. Svarbu, kad šios bioetanolio gamybos metu gautos atliekos būtų saugios pašarų gamybai. Taigi, tiriant fuzariozės pažeistų salyklinių grūdų panaudojimo bioetanolio gamyboje galimybes, tikslinga ištirti skirtingų biotechnologinių priemonių – grūdinės žaliavos fermentinės hidrolizės ir alkoholinės fermentacijos – įtaką bioetanolio gamybos efektyvumo didinimui ir mikotoksinų kiekio gamybos atliekose (žlaugtuose) mažinimui.

Vienintelis patikimas būdas užkirsti kelią salyklinių grūdų užteršimui mikotoksinais yra užtikrinti, kad grūduose nesidaugintų mikroskopiniai grybai. Todėl vartotojams nuo mikotoksinų apsaugoti būtina turėti gerą jų nustatymo ir kontrolės grūduose sistemą. Pasaulinėje praktikoje mikotoksinams nustatyti dažniausiai taikomi daug darbo ir laiko reikalaujantys pamatiniai cheminiai, imunofermentiniai ir fizikiniai mikotoksinų analizės metodai. Kita vertus, iki šiol neišspręsta mėginių paėmimo mikotoksinų analizei problema. Įvertinus tai, kad mikotoksinų nustatymo metodai yra sudėtingi ir brangūs, salyklinių grūdų perdirbimo procese išskirtinis dėmesys turi būti skiriamas inovatyviems metodologiniams sprendimams, kurie leistų greitai ir pigiai aptikti mikotoksinus žaliavoje neimant mėginių.

Kita vertus, mikrobiologinės taršos kontrolė salyklinių grūdų perdirbimo grandinėje siejama su kompleksinių technologinių sprendimų taikymu ne tik vystant naujus mikroskopiniais grybais užkrėstų grūdų aptikimo metodus, bet ir kuriant natūralias salyklinių grūdų apdorojimo (detoksikacijos) biopriemones.

Pirmoji tyrimų kryptis siejama su šiuo metu šalyje vystomu akustiniu metodu ir technika jam realizuoti. EUREKA ITEA2 projektą ACOUSTICS vertinę ekspertai pabrėžė, kad akustika pirmą kartą taikoma grūdų saugos monitoringui, ir pažymėjo idėjos novatoriškumą ir svarbą vartotojo sveikatingumo užtikrinimui. Tačiau iki šiol nėra tirta *Fusarium* spp. mikroskopinių grybų ir jų metabolitų įtaka salyklinių grūdų cheminei sudėčiai ir technologinėms savybėms. Be to, stokojama informacijos apie *Fusarium* spp. užterštų grūdų mikrostruktūros pokyčių ryšį su vystomo akustinio metodo vertinimo parametrais. Kita vertus, labai svarbu užtikrinti tinkamas salyklinių grūdų laikymo sąlygas. Laikomų grūdų gedimas siejamas su tam tikrais išskirtiniais požymiais: puvimo kvapu, padidėjusia grūdų masės temperatūra, išsiskiriančio anglies dioksido ($CO_2$) koncentracijos padidėjimu. Tolydinės $CO_2$ kontrolės technika pagrįsta šio parametro fiksavimu laikomų grūdų masėje. Ji leidžia grūdų gedimą nustatyti tiksliai ir daug anksčiau nei vykdant temperatūros ir drėgmės monitoringą. Toks techninis sprendimas efektyviausias talpyklose, turinčiose aktyviosios ventiliacijos sistemą. Grūdų saugyklose nesant ventiliacijos sistemų, toks $CO_2$ monitoringas grūdų laikymo metu yra probleminis dėl skirtingų dujų migracijos laikomuose grūduose ypatumų. Siekiant padidinti šio metodo tikslumą ir pritaikomumą skirtingų technologinių parametrų grūdų laikymo kontrolei, pvz., neventiliuojamose talpyklose, aktualu vystyti techninius sprendimus, leidžiančius stebėti $CO_2$ koncentracijos pokyčius laikomų grūdų masėje.

Antra vertus, tarptautinėje praktikoje didelio susidomėjimo kaip grūdinės žaliavos detoksikacijos priemonė sulaukė biotechnologiniai preparatai, pagaminti naudojant antimikrobinėmis savybėmis pasižyminčius mikroorganizmus. Šalyje tokie bakteriocinus gaminantys mikroorganizmai buvo išskirti iš ruginių raugų ir sėkmingai pritaikyti maisto pramonėje (FERMFOOD projektas). Antimikrobinį poveikį *Fusarium* spp. mikroskopiniams grybams pademonstravo su bakteriocinus gaminančiomis pieno rūgšties bakterijomis (PRB) pagaminti bioproduktai, kuriais buvo apdorotos kviečių, miežių ir kukurūzų javų sėklos (BIOEKOTECH projektas). Manoma, kad naujų antimikrobinėmis savybėmis pasižyminčių bioproduktų gamybos technologijų kūrimas ir pritaikymas galėtų padidinti salyklo gamybos efektyvumą ir sumažinti *Fusarium* spp. mikroskopinių grybų ir jų gaminamų mikotoksinų kiekį perdirbamoje salyklinėje žaliavoje. Taip pat labai svarbu įvertinti sėklos dygimo metu vykstančius biopreparatais apdorotų salyklinių grūdų baltymų pokyčius, atliekant baltymų frakcionavimą pagal molekulinę masę. Tai leistų geriau suprasti salyklinių grūdų dygimo metu vykstančius biocheminius procesus ir parinkti optimalius

technologinius salyklo gamybos parametrus.

Sparčiai plečiasi ir kitų ekologinių priemonių, pvz., ozono, kaip alternatyvos biotechnologinėms grūdų užterštumo mažinimo priemonėms, panaudojimo grūdinėje žaliavoje esančių mikotoksinų koncentracijai sumažinti galimybės. Ozonas yra gana palankus aplinkai, jį naudojant nesusidaro toksinių liekanų. Vis dėlto ozono antimikrobinis efektyvumas priklauso nuo kelių veiksnių: panaudoto kiekio, mikotoksinų rūšies ir koncentracijos, įvairių aplinkos veiksnių, pvz., grūdų masės temperatūros, drėgmės ir paviršiaus savybių, todėl ozono panaudojimo grūdinės žaliavos saugai užtikrinti galimybių tyrimą būtina plėtoti.

**Darbo tikslas**

Sukurti greitus ir efektyvius metodus *Fusarium* spp. mikroskopinių grybų pažeistiems grūdams aptikti, o šių grybų gaminamiems mikotoksinams detoksikuoti ir fermentacijos proceso efektyvumui didinti panaudoti natūralias ir ekologiškas priemones – pieno rūgšties bakterijų bioproduktus ir ozoną.

**Darbo uždaviniai**

1. Nustatyti ryšį tarp *Fusarium* spp. gaminamo mikotoksino deoksinivalenolio (DON) koncentracijos ir pažeistų salyklinių grūdų cheminės sudėties bei mikrostruktūros pokyčių.
2. Nustatyti ryšį tarp salyklinių kviečių grūdų užterštumo *Fusarium* spp. gaminamu mikotoksinu deoksinivalenoliu lygio ir akustinio signalo parametro verčių.
3. Įvertinti naujus techninius sprendimus, leidžiančius stebėti $CO_2$ koncentracijos pokyčius laikomoje *Fusarium* spp. pažeistoje kviečių grūdų masėje.
4. Nustatyti ozonavimo įtaką mikotoksinų koncentracijos perdirbamoje salyklinėje žaliavoje mažinimui.
5. Įvertinti antimikrobinėmis savybėmis pasižyminčių pieno rūgšties bakterijų bioproduktų, kultivuotų sūrių išrūgų permeato mitybinėje terpėje, įtaką susidariusių metabolizmo produktų kiekiui, salyklo gamybos efektyvumo didinimui ir mikotoksinų koncentracijos perdirbamoje salyklinėje žaliavoje mažinimui.
6. Nustatyti pieno rūgšties bakterijų bioproduktų poveikį *Fusarium* spp. pažeistų salyklinių kviečių grūdų baltyminių medžiagų kiekybinei sudėčiai grūdų dygimo metu, atliekant baltymų frakcionavimą pagal molekulinę masę.
7. Įvertinti skirtingų biotechnologinių priemonių – pieno rūgšties bakterijų ir grūdinės žaliavos fermentinės hidrolizės – panaudojimą fuzariozės pažeistų miežių grūdų alkoholinės fermentacijos efektyvumui didinti ir mikotoksinų kiekiui gamybos atliekose (žlaugtuose) mažinti.

**Mokslinio darbo naujumas**

Išbandytas ir pritaikytas pagal EUREKA ITEA2 projektą ACOUSTICS sukurtas akustinis spektrometras, skirtas greitai ir bekontakčiu būdu kviečių grūdų užterštumo *Fusarium* spp. gaminamu mikotoksinu DON lygiui nustatyti.

Salyklinių kviečių grūdų biologinei taršai – *Fusarium* spp. gaminamų mikotoksinų koncentracijai – sumažinti panaudoti PRB bioproduktai ir ozonas.

Ištirtos pieno perdirbimo atliekų (sūrių išrūgų permeato) panaudojimo antimikrobiniams PRB bioproduktams gaminti ir *Fusarium* spp. pažeistų salyklinių grūdų sveikumui ir daigumui didinti galimybės.

Ištirtas PRB bioproduktų poveikis *Fusarium* spp. pažeistų salyklinių kviečių grūdų baltyminių medžiagų kiekybinei sudėčiai grūdų dygimo metu.

**Teorinė ir praktinė darbo vertė**

Pritaikyta akustinė technika, leidžianti greitai ir efektyviai aptikti *Fusarium* spp. pažeistų kviečių grūdų užterštumą.

Įrodyta, kad pieno perdirbimo šalutinis produktas – sūrių išrūgų permeatas – yra tinkama terpė gaminti PRB bioproduktams, kurie pasižymi antimikrobiniu poveikiu *Fusarium* spp. bei jų gaminamam mikotoksinui DON ir padidina salyklo gamybos efektyvumą.

Nustatyta, kad ozonavimas sumažina *Fusarium* spp. gaminamų mikotoksinų kiekį salykliniuose kviečių grūduose.

PRB, fermentiniai preparatai ir mielės, naudotos *Fusarium* spp. pažeistos miežių grūdinės žaliavos fermentacijos metu, padidina bioetanolio išeigą ir sumažina *Fusarium* spp. gaminamo mikotoksino DON koncentraciją žlaugtuose.

**Ginamieji disertacijos teiginiai**

1. Kviečių grūdų pažeidimui įvertinti galima naudoti akustinę techniką, leidžiančią greitai ir tiksliai aptikti kviečių grūdų pažeidimą, kuris stipriai koreliuoja su kviečių grūduose aptinkamo *Fusarium* spp. gaminamo mikotoksino DON koncentracija.
2. PRB bioproduktai, kultivuoti sūrių išrūgų permeato terpėje, mažina mikroskopinių grybų infekciją kviečių grūdų paviršiuje ir pasižymi detoksikuojamuoju poveikiu *Fusarium* spp. gaminamiems mikotoksinams.
3. PRB, fermentiniai preparatai ir mielės, naudotos *Fusarium* spp. pažeistos miežių grūdinės žaliavos fermentacijos metu, padidina bioetanolio išeigą ir sumažina *Fusarium* spp. gaminamo mikotoksino DON koncentraciją žlaugtuose.
4. Ozonas pasižymi detoksikuojamuoju poveikiu *Fusarium* spp. gaminamiems mikotoksinams.

## IŠVADOS

1. Ištirta *Fusarium* spp. užkrėstų grūdų cheminė sudėtis, mikrostruktūra ir technologinės savybės:
1.1. Miežių ir kviečių grūduose, didėjant DON koncentracijai, krakmolo kiekis mažėjo, o fermentų (amilolitinių, ksilanolitinių ir proteolitinių) aktyvumas didėjo.
1.2. Užkrėstuose grūduose nustatyti šie mikrostruktūros pokyčiai: plonesnės ir susiraukšlėjusios grūdų sienelės, destruktūrizuoti ir formą praradę krakmolo grūdeliai, sudarę pavienius aglomeratus, rodantys galimos hidrolizės požymius, ir padidėję tarpai tarp krakmolo granulių.
1.3. *Fusarium* spp. tarša turėjo neigiamos įtakos kviečių grūdų technologinėms savybėms. Sumažėjo 1000 grūdų masė ir nuo 93,6 % iki 37 % sumažėjo jų daigumas.
2. Sukurtas ir pritaikytas akustinis spektrometras mikotoksinams kviečių grūduose aptikti:
2.1. Nustatyta patikima tiesinė priklausomybė ($r$ = 0,9013, $p \leq$ 0,05) tarp DON koncentracijos, nustatytos akustiniu metodu (DON (Acoust)), ir DON koncentracijos, nustatytos ELISA metodu (DON (ELISA)).
2.2. Nustatytas akustinio metodo pakartojamumo santykinis standartinis nuokrypis (SSN) skirtingam DON koncentracijos lygiui pateko į mažiau nei 20 % gero atsikartojamumo intervalo ribas, nustatytas pagal CEN/TR 16059:2010. Metodo patikimumo įvertinimo parametrai rodo, kad akustiniu metodu galima nustatyti DON kiekį kviečių grūduose.
3. Nustatytas ryšys tarp laikomuose grūduose susidariusios $CO_2$ koncentracijos ir grūdinės žaliavos užkrėstumo (DON) laipsnio, grūdų drėgnio bei grūdų stambumo:
3.1. Užkrėstuose kviečių grūduose (DON – 800 μg/kg), palyginti su sveikais grūdais (DON – 0 μg/kg), nustatytas padidėjęs $CO_2$ kiekis esant padidėjusiam grūdų drėgniui (18,5 % ir 24,5 %), o esant standartiniam grūdų drėgnumui (14,5 %) reikšmingo $CO_2$ koncentracijos skirtumo tarp šių grūdų nenustatyta.
3.2. Nustatyta, kad $CO_2$ stambiuose grūduose išsiskiria intensyviau nei smulkiuose.
3.3. Laikomoje grūdų masėje su *Fusarium* spp. taršos židiniu fiksuota didesnė $CO_2$ koncentracija.
4. Didžiausias detoksikuojamasis *Fusarium* spp. grybais užkrėstų salyklinių kviečių grūdų ozonavimo efektas priklausė nuo poveikio trukmės: vidutiniškai didžiausiu detoksikuojamuoju poveikiu pasižymėjo 130 min. trukmės pažeistų kviečių apdorojimas ozonu. Mikotoksinų DON, ZEN, T-2 ir HT-2 koncentracija, palyginti su kontroliniu variantu, sumažėjo atitinkamai 25 %, 48,7 %, 65 % ir 82,8 %.

5. Ištirtas PRB bioproduktų panaudojimas salykliniams grūdams apdoroti. Nustatyta:

5.1. Sūrių išrūgų permeatas yra tinkama fermentacinė terpė PRB bioproduktams gaminti, joje gerai dauginasi PRB ir kaupiasi antimikrobiniai metabolizmo produktai (pieno ir acto rūgštis).

5.2. PRB bioproduktais apdorotuose *Fusarium* spp. užkrėstuose kviečių grūduose DON koncentracija sumažėjo nuo 16,59 iki 66,86 %. Didžiausiu antigrybeliniu poveikiu pasižymėjo bioproduktai, fermentuoti su *L. bulgaricus* (Lb) (95 %), *L. sakei* (Ls) (93,3 %) ir *P. acidilactici* (Pa7) (91,5 %).

5.3. *Fusarium* spp. užkrėstų grūdų daigumas po apdorojimo Pa7 ir Pp10 padermėmis, palyginti su kontroliniu variantu, padidėjo vidutiniškai 7,5 %.

6. Tyrimais įrodyta, kad apdorojimas PRB bioproduktu neturi įtakos *Fusarium* spp. pažeistų salyklinių grūdų baltyminių medžiagų kiekybinei sudėčiai grūdų dygimo metu.

7. Ištirtas biopriemonių panaudojimas *Fusarium* spp. užkrėstų miežių grūdų fermentacijos efektyvumui didinti. Nustatyta:

7.1. DON koncentracijai fermentuojamuose miežių grūduose didėjant nuo 120 iki 1225 µg/kg, susidariusio etanolio kiekis sumažėjo atitinkamai nuo 7,5 iki 12,2 %, o susidariusių aukštesnės eilės alkoholių kiekis padidėjo nuo 21 iki 158,43 %, palyginti su nepažeistais grūdais (DON – 0 µg/kg).

7.2. Didžiausia etanolio koncentracija (74,6 g/l) gauta, kai miežių grūdų biomasė fermentuota su 3450 XU/kg ksilanazės. Taip pat ksilanazė susidariusių aukštesnės eilės alkoholių kiekį distiliate sumažino vidutiniškai iki 23,9 %.

7.3. Naudojant Ls ir *K. marxianus* var. *bulgaricus* mielių derinį, nustatyta didžiausia susidariusio bioetanolio išeiga tarp visų tirtų PRB ir mielių derinių – ji buvo 87 % teoriškai nustatytos biomasės išeigos.

7.4. Pažeistų miežių fermentacijai panaudojus Ls ir *K. marxianus* var. *bulgaricus* mielių derinį, DON koncentracija žlaugtuose, palyginti su pradine žaliava, sumažėjo 55 %.

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