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# ZEARALENONE BIODEGRADATION BY THE *LACTOBACILLUS* SPP. AND *BACILLUS* SPP. *IN VITRO*

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## ABSTRACT

**In this study, the biodegradation of zearalenone (ZEN) by cell suspensions of various *Lactobacillus* species (*Lb. fermentum* 2I3 (L1), *Lb. reuteri* L26 (L3), *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5), *Lb. plantarum* CCM 1904 (L6)), *Bacillus subtilis* CCM 2794 (Bs), and *Bacillus licheniformis* CCM 2206 (Bl); was investigated *in vitro*. All lactobacilli cell suspensions showed very good degradation efficiency (57.9—100 %) for zearalenone at the concentration 0.01 ppm. At higher concentrations of zearalenone, their biodegradation activity decreased significantly (0—13.9 %). *Bacillus subtilis* CCM 2794 was able to degrade zearalenone at concentrations of: 0.01 ppm (100 %), 0.1 ppm (74.5 %), and at higher concentrations of ZEN (1 ppm; 10 ppm), the degradation was 11.7 % and 0 %, respectively. For *Bacillus licheniformis* CCM 2206, no biodegradation of zearalenone was observed at the concentration of 10 ppm, but slight degradation (4.5—8.8 %) was found at lower zearalenone concentrations.**

**Key words: *Bacillus* spp.; degradation; *Lactobacillus* spp.; mycotoxins**

## INTRODUCTION

Mycotoxins are a large group of secondary metabolites of microscopic filamentous fungi. Fungal secondary metabolites threaten human and animal health worldwide by contaminating food and feed. More than 300 kinds of mycotoxins with different structures have been identified so far [5, 18]. Mycotoxins have no biochemical significance for fungal growth or development [19]. Mycotoxin production depends on environmental factors, such as: relative humidity, ambient pH, temperature and season. Other factors that affect mycotoxin production include: the presence of oxygen, thermal damage, mechanic damage to grain integrity during crop harvesting, damage to grain integrity by insects, plant variety, plant density, soil structure, fungal inoculum quality and vegetation stage [4]. One of the most important mycotoxins in the world is zearalenone (ZEN). It is synthesized by the microscopic

filamentous fungi *Fusarium* spp. (mainly by *F. graminearum, F. culmorum* and *F. sporotrichioides*) belonging to the field fungi.

Chemically, zearalenone is a 6-(10-hydroxy-6-oxy-trans-1-undecenyl)-beta-resorcylic acid lactone with the general formula C18H22O5. ZEN is a non-steroidal oestrogen mycotoxin which is biosynthesized via the polyketide pathway. Optimal conditions for the production of ZEN by fungi are characterized by temperatures between 20 and 25 °C and humidity above 20 %. The high levels of zearalenone are frequently found in countries with a mild and wet climate [15]. Zearalenone has immunotoxic, hepatotoxic, xenogenic, hematotoxic and other effects.

The most significant negative effect of zearalenone is its strong estrogenic activity. This activity in living organisms depends on the immune status of the organism and the state of the reproductive system. The estrogenic effects of zearalenone include: reduced fertility or infertility, vaginal prolapse, vulvar swelling and breast enlargement in females, feminization of testicular atrophy, and enlargement of the mammary glands in males [1]. Pigs and ruminants are the most sensitive to the effects of ZEN while the most resistant ones are birds, such as chickens and poultry [13].

In humans, zearalenone toxicity is predominantly chronic. ZEN and its metabolites can stimulate the growth of mammary gland cells and may be involved in breast cancer. However, ZEN is included in noncarcinogenic agents to humans, Group 3, according to the International Agency for Research on Cancer (IARC) [1, 3].

Various methods are employed to eliminate mycotoxins. Decontamination methods are divided into three basic groups: physical, chemical and biological [12]. The biological methods based on the degradation and transformation of mycotoxins by different strains of bacteria and yeasts are currently preferred [14]. Yeasts and lactic acid bacteria are involved in the biological adsorption of mycotoxins and prevent their absorption from the digestive tract into the blood of humans and other animals [10]. *Lactobacillus* spp. has a high ability to suppress the growth of microscopic filamentous fungi and inhibit the production of mycotoxins [8]. Microorganisms such as *Bacillus* spp. which are isolated from soil and other environments have been reported to be able to degrade mycotoxins [18]. *Bacillus* strains produce and secrete large quantities of extracellular enzymes, and are under investigation of mycotoxin biodegradation. However, these enzymes have been reported to degrade only a certain type of mycotoxins [10, 16]. The aim of this research was to determine the ability of cell suspensions by various species of the genera *Lactobacillus* and *Bacillus* (*Bacillus subtilis* and *Bacillus licheniformis*) in zearalenone degradation *in vitro*.

## MATERIALS AND METHODS

Biodegradation of zearalenone (Sigma Aldrich, Schnelldorf, Germany) was performed by selected strains of *Lactobacillus* spp. and *Bacillus* spp. The experiment was set up similar to that described by Harkai et al. [9].

### Tested microorganisms

In the experiments, seven strains of microorganisms were used (Table 1). Strains of *Lactobacillus plantarum* CCM 1904, *Bacillus subtilis* CCM 2794 and *Bacillus licheniformis* CCM 2206 were purchased from the Czech Collection of Microorganisms (Masaryk University, Faculty of Science, Institute of Experimental Biology, Brno, Czech Republic). Other strains of the genus *Lactobacillus* were acquired from the Department of Microbiology and Immunology of the University of Veterinary Medicine and Pharmacy in Košice.

**Table 1. Tested microorganisms**

| Species | Abbreviations used |
|---|---|
| *Bacillus subtilis* CCM 2794 | Bs |
| *Bacillus licheniformis* CCM 2206 | Bl |
| *Lactobacillus plantarum* CCM 1904 | L6 |
| *Lactobacillus plantarum* L81 | L4 |
| *Lactobacillus fermentum* 2I3 | L1 |
| *Lactobacillus reuteri* 2/6 | L5 |
| *Lactobacillus reuteri* L26 | L3 |

### Cultivation media

Commercial MRS medium (HiMedia, Laboratories Pvt. Ltd., Mumbai, India) was used to cultivate lactobacilli strains. *Bacillus* spp. strains were cultured on Medium B10 prepared according to a recipe obtained from the Czech Collection of Microorganisms (Masaryk University, Faculty of Science, Institute of Experimental Biology, Brno, Czech Republic) (Table 2).

**Table 2. Composition of nutrient media in a volume of 1000 ml of distilled water**

| | |
|---|---|
| **MRS broth** | Yeast extract 5 g, meat broth 10 g, peptone 10 g, glucose 20 g, Tween 80 5 ml, $KH_2PO_4$ 2 g, sodium acetate 5 g, ammonium citrate 2 g, $MgSO_4 . 7H_2O$ 0.2 g, $MnSO_4 . 4H_2O$ 0.05 g |
| **MRS agar** | Yeast extract 5 g, meat broth 10 g, peptone 10 g, glucose 20 g, Tween 80 5 ml, KH2PO4 2 g, sodium acetate 5 g, ammonium citrate 2 g, $MgSO_4 . 7H_2O$ 0.2 g, $MnSO_4 . 4H_2O$ 0.05 g, agar 12 g |
| **Medium B10 broth** | Peptone 5 g, meat broth 3 g, $MnSO_4 . H_2O$ 0.01 g |
| **Medium B10** | Peptone 5 g, meat broth 3 g, $MnSO_4 . H_2O$ 0.01 g, agar 20 g |

### Preparation of cell suspensions of *Lactobacillus* spp. and *Bacillus* spp.

Twenty-four hours old cultures of *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) grown on B10 agar at 35 °C were used to prepare the cell suspensions. Using a sterile inoculation loop (mesh volume 10 µl), the colonies of bacteria were picked up and transferred to 50 ml of B10 broth in an Erlenmeyer flask. Cell suspensions, from individual lactobacilli, strains grown under anaerobic conditions using a Gas pack system on MRS agar at 35 °C for 48 h, were prepared by inoculating 50 ml of MRS broth. Media inoculated with *Lactobacillus* spp. and *Bacillus* spp. were incubated at 35 °C under aerobic conditions by shaking the Erlenmeyer flasks on a horizontal shaker (Orbital Shaker—Biosan) at 170 rpm. After 24 hours, cell suspensions of *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) were centrifuged for 20 minutes at 6000 rpm and the supernatants were removed. The cell pellets were then washed twice with 5 ml of PBS buffer and again centrifuged under the same conditions. Subsequently, the cell pellets were resuspended and adjusted with saline solution/PBS buffer to a density of McFarland 4, (corresponding to 108 CFU.ml$^{-1}$) using a densitometer.

### Zearalenone biodegradation testing procedure

The stock solution of 5000 ppm (5000 mg.l$^{-1}$) of zearalenone was prepared by dissolving the powder (25 mg) in 100 % of DMSO (dimethyl sulfoxide). This solution was used to prepare zearalenone concentrations of 10 ppm (10 mg.l$^{-1}$), 1 ppm (1 mg.l$^{-1}$), 0.1 ppm (0.1 mg.l$^{-1}$), and 0.01 ppm (0.01 mg.l$^{-1}$) by dissolving in a liquid cultivation medium (MRS broth for *Lactobacillus* spp. and B10 broth for *Bacillus* spp.) so that the final DMSO concentration was 0.2 %. The test was performed in test tubes. 100 µl of cell suspension of the strains were added to 4.95 ml of medium containing various concentrations of zearalenone. The tubes containing only the strains in the cultivation medium (without zearalenone) served as a negative control, thus monitoring their multiplication. The positive control was represented by tubes with individual concentrations of zearalenone in the culture medium, without inoculum of the bacterial suspension. The samples were incubated at 35 °C depending on the strains 24 h for bacilli and 48 h for lactobacilli. The residual zearalenone was extracted from the samples using Neocolumn for Zearalenone (Neogen Corporation, USA). An ELISA method was used to determine the zearalenone, using the Veratox for Zearalenone ELISA kit (Neogen Corporation, USA). Based on the measured concentrations, the percentage of biodegradation was calculated according to the formula:

$$\% \text{ biodegradation} = \frac{C_{PC} - C_S}{C_{PC}} \times 100\ \%$$

where: $C_s$— zearalenone concentration in the sample
$C_{PC}$—zearalenone concentration in the positive control

## RESULTS

The efficacy of *Lactobacillus* spp. and *Bacillus* spp. cell suspensions in the biodegradation of zearalenone at the various concentrations are presented in Table 3. As the results demonstrated, the lactobacilli cell suspensions possessed a very good ability to degrade zearalenone at lower concentrations (0.01 ppm), with an efficacy in the range of 57.9—100 %. However, at higher ZEN concentrations, their biodegradation efficiency decreased significantly (0—13.9 %). A different ability of zearealenone biodegradation was found for the bacilli. The capability of *Bacillus subtilis* to degrade zearalenone at concentration of 0.01 ppm reached 100 % and 74.5 % at 0.1 ppm, but at higher ZEA concentrations, its biodegradation efficacy was insufficient (11.7 % and 0 %, respectively). *Bacillus licheniformis* CCM 2206 was not effective at the highest tested zearalenone concentration (10 ppm), and also at decreasing ZEA concentrations its degradation ability was very low (4.5—8.8 %).

**Table 3. Biodegradation efficacy (%) of cell suspensions of *Lactobacillus* spp. and *Bacillus* spp. at different zearalenone concentrations**

| Strain | ZEN concentrations | | | |
|---|---|---|---|---|
| | 10 ppm | 1 ppm | 0.1 ppm | 0.01 ppm |
| L1 | 5.4 | 3.9 | 12.5 | 57.9 |
| L3 | 1.5 | 1.7 | 0 | 85.6 |
| L4 | 2.8 | 0 | 13.9 | 100 |
| L5 | 2.8 | 1.9 | 3.1 | 100 |
| L6 | 0 | 6.5 | 8.5 | 100 |
| Bs | 0 | 11.7 | 74.5 | 100 |
| Bl | 0 | 4.5 | 6.09 | 8.8 |

L1—*Lb. fermentum* 2I3; L3—*Lb. reuteri* L26; L4—*Lb. plantarum* L81; L5—*Lb. reuteri* 2/6; L6—*Lb. plantarum* CCM 1904; Bs—*Bacillus subtilis* CCM 2794; Bl—*Bacillus licheniformis* CCM 2206; ZEN—zearalenone

## DISCUSSION

Biodegradation is one of the most specific and environmentally friendly methods in reducing the possible mycotoxins from food and feed. In general, *Lactobacillus* spp. and *Bacillus* spp. are considered safe strains for detoxification of zearalenone in feed [18]. When testing the detoxifying effects of *Lactobacillus* spp. and *Bacilllus* spp. cell suspensions, the highest biodegradation ability (100 %) was found in *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5), *Lb. plantarum* CCM 1904 (L6) and *Bacillus subtilis* CCM 2794 (Bs) at the zearalenone concentration of 0.01 ppm. However, at a zearalenone concentration of 1 ppm, the degradation was significantly lower (0—11.7 %). Z h a o et al. reported that ZEN (100 $\mu g.l^{-1}$) decreased by 45 % after 48 hours of exposure to *Lactobacillus plantarum* [20]. Similarly, A d u n p h a t c h a r a p h o n et al. [2] published that ZEN degradation at concentration 0.2 $\mu g.ml^{-1}$ using *Lactobacillus* spp. were in the range of 0.5—23 %. Probably the main cause of zearalenone reduction are bacterial cell proteins and lipids [2]. *Lactobacillus* spp. cell wall is composed of glycopolymers and proteins and it consists of a dense peptidoglycan with proteins, polysaccharides and teichoic acids. The surface structure of a bacterial cell is affected by the difference between sugars and amino acids in the glycopolymer or protein structures. This difference in surface structure plays an important role in the ability of *Lactobacillus* spp. to detoxify ZEN [6]. H s u et al. [11] tested the degradation of ZEN by *Bacillus licheniformis* CK1 and recorded a reduction of 70 % (from 5 $\mu g.ml^{-1}$ to 0.5 $\mu g.ml^{-1}$). However, at higher ZEN concentrations (50 and 200 $\mu g.ml^{-1}$) the degradation effect was only 60 % and 10 %, respectively [11]. *B. licheniformis* has outstanding probiotic properties (acidic tolerance, bile salt tolerance, adherence capability, and anti-pathogenic activities), therefore, it could also be used to improve the digestibility of nutrients in feed [11, 18]. T i n y i r o et al. found that *B. subtilis* 168 was more efficient in the removal of zearalenone from liquid medium, and that more than 75 % of ZEN was eliminated after incubation [17]. C h o et al. reported that *B. subtilis* strain degraded 99 % of ZEN in a liquid medium [7]. According to H a t h o u t and A l y, decontamination of zearalenone is never complete unless the presence of its oestrogenic analogues is ruled out [10].

## CONCLUSIONS

The use of microorganisms or their enzymes to detoxify mycotoxins is a new option in the production of healthy food and feed. This study points to the potential use of the cell suspensions of non-pathogenic microorganisms (*Lactobacillus* spp. and *Bacillus* spp.) for zearalenone degradation. These strains could be used as innocuous detoxifying agents. These partial results also indicate the further direction of scientific research. Furthermore, it will be necessary to investigate the properties of non-pathogenic microorganisms capable of inactivating mycotoxin in feed and food.

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