PRELIMINARY COMMUNICATION

Adhesion of Zearalenone to the Surface of Lactic Acid Bacteria Cells

Domagoj Čvek*, Ksenija Markov, Jadranka Frece, Maja Friganović, Lejla Duraković, Frane Delaš

Faculty of Food Technology and Biotechnology, University of Zagreb, Laboratory for General Microbiology and Food Microbiology, Pierottijeva 6, 10000 Zagreb, Croatia

Summary

Zearalenone is mycotoxin harmful to the health of humans and animals and is present in food and feed. Therefore, last few decades, great attention is paid to the development of biological detoxification methods with reference to the application of lactic acid bacteria in binding and removal of mycotoxins from food and feed. The aim of this work was to investigate the ability of lactic acid bacteria Lactobacillus rhamnosus GG (ATCC 53103) and Lactobacillus plantarum A1 to bind zearalenone unto the cell surface. The results showed that Lactobacillus rhamnosus GG (ATCC 53103) and Lactobacillus plantarum A1 can bind significant concentration of zearalenone, depending on the bacteria concentration in the medium and the incubation time. However, with longer time of incubation part of bounded mycotoxins were released back into the medium which indicates that the reaction of adhesion of toxin to the bacterial cells is reversible.

Determination of zearalenone concentration was performed by competitive ELISA test.

Keywords: zearalenone, Lactobacillus plantarum A1, Lactobacillus rhamnosus GG (ATCC 53103)

Introduction

Zearalenone is a no steroidal estrogen toxin, often called phytoestrogen, produced by the fungi from genus *Fusarium* including *F. roseum*, *F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. equiseti*, *F. semitectum*, *F. oxysporum* and *F. tricinctum* (Betina, 1989; Bennett and Klich, 2003). These fungi grow on agricultural crops such as maize, rice, wheat, barley, sesame, oat and soybean (D'Mello et al., 1999). Factors affecting the growth of these moulds and zearalenone biosynthesis are temperature, humidity, substrate, and mould species. The optimum temperature of zearalenone biosynthesis is between 5 and 25 °C with 16 % humidity (Gajecki et al., 2010).

Zearalenone (6-(10-hydroxy-6-oxo-trans-1-undecinil) Resorcylic acid lactone) has a stable chemical structure and is not subject to degradation during storage, treatment and processing of crops in which is present. It is soluble in alkaline solutions, ether, benzene, acetonitrile and ethanol, while it is insoluble in water (Gajecki et al., 2010).

During oral intake, zearalenone is quickly and efficiently absorbed through the gastrointestinal tract, so that up to 85 % of the initial dose of zearalenone could be determined in blood. After intake, before or after absorption, zearalenone can be biotransformed by the enzymes to derivatives subsequently conjugated with glucuronic acid which then could be distributed within the organism (Kuiper-Goodman et al., 1987, Zinedine et al., 2007).

Zearalenone influences reproductive system, and changes caused by zearalenone intake can be divided into three categories: 1. disorders of the sexual cycle, including sterility; 2. disturbances in the physiology of pregnancy, including spontaneous abortion; 3. lesions of the female reproductive organs and mammary glands (Golinski et al., 1988).

Also, one should not exclude the possibility of influence of this estrogenic factor on the formation of ovarian cancer, uterine and breast cancer in animals.

As a result of growing awareness of risks to human health caused by mycotoxins, 35 % of world countries have introduced guidelines and regulations for mycotoxins (FAO, 1997).

Presence of mycotoxicogenic moulds and/or mycotoxins in food is potentially dangerous to the health of the people and also represents economic problem. The physical and chemical methods to control the presence of organisms that produce micotoxins and their toxins are developed, but an accurate, reliable and effective strategy for elimination of mycotoxins has not yet been found. Therefore, the development of such strategies and appropriate methods are of primary importance.

Strains of lactic acid bacteria (LAB) have been used in the production of fermented foods as starter cultures and aroma producers. The main role of such cultures is to produce organic acids such as lactic acid trough fermentation and to increase both the shelf life of the product and to alter its sensory properties (Peltonen et al., 2001; Frece et al., 2009; Babić et al., 2011). In the last fifteen years researches have been trying to investigate if the LAB also have, among other proven desirable properties, the ability to bind mycotoxins from food and feed.

Namely, some authors reported that specific strains from genus *Lactobacillus* can reduce concentration of mycotoxins present in food and feed (Dalié et al., 2010; Markov et al., 2010).

Partial removal of mycotoxins involves physical binding of the toxin probably to the bacterial cell wall or cell wall components (Peltonen et al., 2001). Such method for removal of mycotoxins in the near future could serve as a good alternative to existing methods of detoxification.

The aim of this study was to investigate the capacity of lactic acid bacteria *Lactobacillus plantarum* A1 and *Lactobacillus rhamnosus* GG (ATCC 53103) for binding of zearalenone (ZEA) *in vitro*.

Materials and methods

Standard of ZEA

Crystalline ZEA was purchased from Sigma (St. Louis, Mo., USA), suspended in benzene-acetonitrile (97/3, vol/vol) to obtain final ZEA concentration of 1 mg /mL.

Corresponding author: dcvek@pbf.hr

Preparation of bacterial cultures inocula

L. rhamnosus strain GG (ATCC 53103) was obtained from Valio Ltd., Helsinki, Finland. *Lactobacillus plantarum* A1 was isolated from cow cheese and stored in Collection of microorganisms from Laboratory of General Microbiology and Food Microbiology of Faculty of Food Technology and Biotechnology, University of Zagreb (Croatia). Lactic acid bacteria were cultivated in 5 mL of de Mann, Rogosa, Sharpe (MRS) broth (Biolife, Italy) at 37 °C for 24 h. The bacterial growth were determined by using MRS agar plates (Biolife, Italy) after 24 hours incubation at 37 °C using traditional plate counting (CFU/mL).

Adhesion of ZEA to the surface of lactic acid bacteria cells

To 20 mL of MRS broth in Erlenmeyer flasks 20 µg/mL of zearalenone was added in aseptic conditions, and media were inoculated by adding suspension of LABs. Final concentration of bacterial cells was 4 $\log_{10}$CFU/mL, 6 $\log_{10}$CFU/mL, or 8 $\log_{10}$CFU/mL. Suspensions were incubated at 37 °C for 72 hours. Supernatant of samples were collected after centrifugation at 0, 24, 48 and 72 h time points. Concentration i.e. percentage of ZEA in supernatant was quantified by ELISA method

Quantification of ZEA by ELISA method

The quantitative analysis of ZEA was performed by using enzyme immunoassay ridascreen zearalenone (Neogen-Veratox 8110-Neogen Europe, Ltd., Scotland, UK). The test kit is sufficient for 48 determinations. The principle of the method is the antigen–antibody reaction. The wells in the microtiter strips are coated with antibodies that are specific to ZEA. After adding different solutions of ZEA standard or sample solutions, the antibody binding sites becomes occupied and its availability is inversely proportional to concentration of ZEA in added solution. Remaining free binding sites will be occupied in the next step, when enzyme labelled toxin (enzyme conjugate is added). The unbound enzyme conjugate is then removed during washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated (room temperature/10 minutes). Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a colour change from blue to red. Intensity of developed red color was determined photometrically at 650 nm (optional reference wavelength P600 nm). The absorption is inversely proportional to the ZEA concentration in the sample.

Statistical methods

All experiments were carried out in triplicate. The results are expressed as mean ± S.D. (standard deviation). The SAS statistical computer package (SAS Institute, Cary, NC, USA) was used to analyze the experimental data.

Results and discussion

This work was done in order to investigate capability of LAB to reduce concentration of ZEA, as a possible alternative to existing physical and chemical methods of detoxification.

The modern trend in the diet includes reduction of synthetic food additives and food production with less undesirable impact on the environment, as well as use of additives or food ingredients of natural origin that contribute to increasing food security (Frece et al., 2010). Therefore, there is great demand for new strategies to prevent contamination of food products and the undesirable effects caused by zearalenone.

In this work the capacity of LAB *Lactobacillus rhamnosus* GG (ATCC 53103), and *Lactobacillus plantarum* A1 to bind zearalenone onto the cell surface was investigated. High percentage of ZEA has been attached to the cells of isolated strain - *L. plantarum* A1 (Table 1). Efficacy of ZEA adhesion onto the LAB cells was monitored by determining concentration of

Table 1. *Effect of incubation time and inoculum size on adhesion of ZEA to the surface of L. plantarum A1 cells*

	% of initial concentration of ZEA bounded to bacterial cells			
	Time of incubation (h)			
L. plantarum A1 $\log_{10}$CFU/mL	[1]0	24	48	72
4	95.24±0.14	69.77±0.17	75.14±0.16	71.13±0.20
6	98.10±0.16	75.00±0.26	78.74±0.22	71.09±0.27
8	99.12±0.25	79.08±0.18	85.67±0.26	77.17±0.21

Values are means ± SD of triplicate
[1] 0 h sample collected after centrifugation

Table 2. *Effect of incubation time and inoculum size on adhesion of ZEA to the surface of L. rhamnosus GG (ATCC 53103) cells*

	% of initial concentration of ZEA bounded to bacterial cells			
	Time of incubation (h)			
L. rhamnosus GG $\log_{10}$CFU/mL	[1]0	24	48	72
4	71.26±0.21	71.26±0.11	62.02±0.19	61.28±0.18
6	81.18±0.24	71.26±0.19	71.09±0.25	65.01±0.18
8	84.71±0.27	75.14±0.22	71.26±0.24	66.30±0.29

Values are means ± SD of triplicate
[1] 0 h sample collected after centrifugation

ZEA in supernatant. The results show that already at the begining of incubation (0 h) between 95 % and 99 % of ZEA from suspension have been attached to the cells of L. plantarum A1, while, at the end of incubation (72 h) between 71 % and 77 % of ZEA, which depends on concentration of LAB cells in the suspension. However, after 24 hours of incubation cells from suspensions released approximately 23 % of ZEA back to the medium. This results indicates that the binding of toxin is reversible process. The concentration of zearalenone in the medium decreases, with longer incubation (48 h and 72 h), and this finding means that ZEA could be repetitively bound and/or released from the bacterial cells.

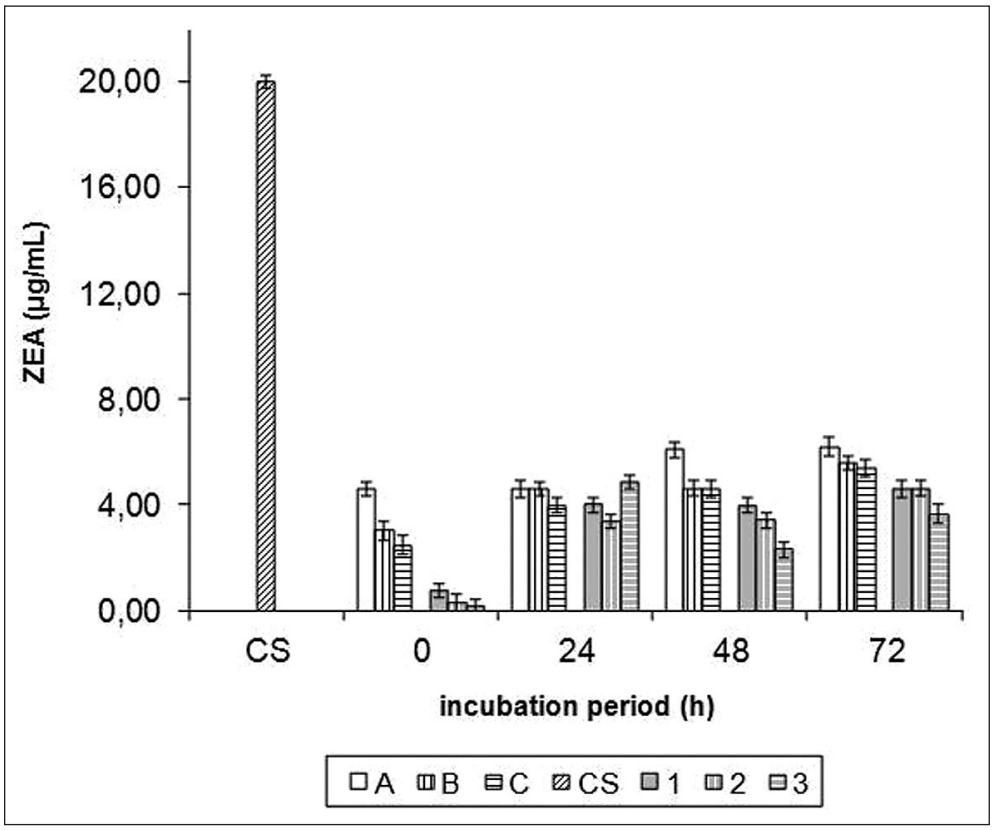

Figure 1. *Concentration of ZEA (µg/mL) determined in supernatant after 72 h of incubation period (A-L. rhamnosus GG (ATCC 53103) 4 log_{10}CFU/mL; B-L. rhamnosus GG (ATCC 53103) 6 log_{10}CFU/mL; C-L. rhamnosus GG (ATCC 53103) 8 log_{10}CFU/mL; 1-L. plantarum A1 4 log_{10}CFU/mL; 2-L. plantarum A1 6 log_{10}CFU/mL; 3-L. plantarum A1 8 log_{10}CFU/mL; CS–control sample/without added LABs)*

Obtained results clearly show that the probiotic strain *L. rhamnosus* GG (ATCC 53103) removed very high concentration of ZEA from the liquid medium during the whole incubation time, which is in agreement with results of authors who have proven that strains of *L. rhamnosus* GG and *L. rhamnosus* Lc705 LC1/3 bind about 80 % and 60 % of ZEA from liquid media (El-Nezami et al., 2002b). Between 71.26 % to 84.71 % of ZEA has been bounded to the cells of *L. rhamnosus* GG at the beginning of incubation, and between 61.28 % and 66.30 % of ZEA after 72 h of incubation (Table 2). Although the binding efficiency of *L. rhamnosus* GG (ATCC 53103) cells is 15 % smaller than the efficiency of *L. plantarum* A1, it is also determined a significant reduction of ZEA in the medium immediately after the toxin is added to the suspension. Between 15 % and 28 % of ZEA has been released from *L. plantarum* A1 and *L. rhamnosus* GG (ATCC 53103) cells during 72 h of incubation in described conditions. These results are in agreement with findings obtained in studies of El-Nezam et al. (2002a) who also proved that the highest concentration of ZEA was bonded to bacterium *Lactobacillus rhamnosus* GG immediately after their mixing. Unlike the results of this study in which the maximum percentage of binding of ZEA to cells of *Lactobacillus rhamnosus* GG (ATCC 53103) was around 85 %, in their study corresponding percentage reached value of 60 %. Also it is noted the release of toxin back to the medium of about 10 %, but after 72 h of incubation, the percentage of ZEA reached same value as after 0 h of incubation. Removal of toxins by using LAB in a liquid medium depends on the concentration of bacteria, so that the highest percentage of bounded ZEA was determined in the medium with the highest concentration of bacteria. Also in the study of El-Nezam et al. (2002a) efficiency of binding of ZEA depended on the concentration of bacteria in the medium and it was noted that most of zearalenone was bounded when LAB was inocculted and final concentration of bacterial cells was between 8–10 log_{10}CFU/mL. Some authors describes differences in the ability of LAB cells to bind mycotoxins due to the different structure of the cell wall, while others support hypothesys that the number of bacteria in the medium is of great importantance (El-Nezami et al., 1998; Pieridis et al., 2000; El-Nezami et al., 2002a, b; Haskard et al., 2001).

The difference in binding of mycotoxins by LAB cells, used in this study (*L. rhamnosus* GG (ATCC 53103) and *L. plantarum* A1), was confirmed also by results of Markov et al. (2010) obtained in studies of binding of aflatoxin M1 (AFM1). The research showed that the probiotic strain *L. rhamnosus* GG (ATCC 53103) significantly removes AFM1 (more than 50 % of its initial concentration), from a milk sample during incubation time, whereas, *L. plantarum* A1 binds relatively small amount of AFM1 ranging from 18.7 % at the beginning of incubation to 28.7 % after 48 hours. Figure 1 shows that the strain *L. plantarum* A1 bind approximately 1–2 µg/mL more ZEA than *L. rhamnosus* GG (ATCC 53103). The biggest difference in the binding of zearalenone by LAB has been observed at the beginning of incubation when the number of cells of the *L. plantarum* A1 in medium was 4 log_{10}CFU/mL. The difference in binding of ZEA by selected strains of LAB is reduced in experiments with cells number of 6–8 log_{10}CFU/mL at the end of incubation (72 h).

In order to develop relatively safe and practical way to remove ZEA from liquid media, numerous studies with specific strains of LAB were carried out (El-Nezami et al., 2002a,b; Niderkorn et al., 2006; Zinedine et al., 2007). The results showed that certain LAB species used in fermented products may offer a new way of removing ZEA from liquid media.

LAB cells posses ability to bind mycotoxins from food and feed, although the binding affinity of particular mycotoxin

to the cells of particular strain of LAB, differs from the affinity of other mycotoxin to cells of the same strain. This can be explained by differences in chemical structure and physicochemical properties of mycotoxins.

Conclusions

The research area of mycotoxin removal by lactic acid bacteria is still underexplored and requires much attention. Based on obtained results, it can be concluded, cell of LAB *Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus plantarum* A1, at 37 °C during 72 hours of incubation in MRS broth, bind ZEA. Efficiency of ZEA removal by its adhesion on bacterial cells depends on the concentration of bacteria in the medium and it is partly reversible process indicating that the binding occurs on the surface of the cell walls. Application of these bacteria may be useful to humans and animals exposed to zearalenone.

References

Babić I., Markov K., Kovačević D., Trontel A., Slavica A., Đugum J., Čvek D., Svetec I.K., Posavec S., Frece J. (2011) Identification and characterization of potential autochthonous starter cultures from a Croatian "brand" product "Slavonski kulen". Meat Science 88, 517-524.

Bennett J.W., Klich M. (2003) Mycotoxins. *Clinical Microbiology Reviews* 16, 497-516.

Betina V. (1989) Mycotoxins: chemical, biological and environmental aspects. Elsevier Science Publishers, Amsterdam, the Netherlands.

Dalié D.K.D., Deschamps A.M., Richard-Forget F. (2010) Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. *Food Control* 21, 370–380.

D'Mello J.P.F., Placinta C.M., Macdonald A.M.C. (1999) *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology* 80, 183-205.

El-Nezami H., Kankaanpaa P., Salminen S., Ahokas J. (1998) Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. *Food and Chemical Toxicology* 36, 321–326.

El-Nezami H. S., Polychronaki N., Salminen S., Mykkänen H. (2002a) Binding rather metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative a-zearalenol. *Applied and Environmental Microbiology* 68, 3545–3549.

El-Nezami H. S., Chrevatidis A., Auriola S., Salminen S., Mykkänen H. (2002b) Removal of common *Fusarium* toxins in vitro by strains of *Lactobacillus* and *Propionibacterium*. *Food Additives & Contaminants* 19, 680–686.

Frece J., Kos B., Svetec I.K., Zgaga Z., Beganović J., Leboš A., Šušković J. (2009) Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. *Journal of Dairy Research* 76, 98-104.

Frece J., Markov K., Kovačević D. (2010) Determination of indigenous microbial populations, mycotoxins and characterization of potential starter cultures in Slavonian kulen. *Meat* XII (2), 92-98.

Gajecki M., Gajecka M., Jakimiuk E., Zielonk L., Obremski L. (2010) Mycotoxins in Food, Feed and Bioweapons. In: Rai M., Varma A, (ed.). *Zearalenone: Undesirable substance*. Springer, London, p. 113-144.

Golinski P., Vesonder R.F., Latus-Zietkiewicz D., Perkowski J. (1988) Formation of fusarenone X, nivalenol, zearalenone, α-trans-zearalenol, β-trans-zearalenol, and fusarin C by *Fusarium crookwellense*. *Applied and Environmental Microbiology* 54, 2147-2148.

Haskard C., El-Nezami G. S., Kankaanpää P. E., Salminen S., Ahokas J. T. (2001) Surface binding of aflatoxin B1 by lactic acid bacteria. *Applied and Environmental Microbiology* 67, 3086–3091.

Kuiper-Goodman T., Scott P.M., Watanabe H. (1987) Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology* 7, 253-306.

Leffers H., Næsby M., Vendelbo B., Skakkebæk N.E., Jørgensen M. (2001) Oestrogenic potencies of zeranol, oestradiol, diethylstilbestrol, bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. *Human Reproduction* 16**,** 1037-1045.

Markov K., Frece J., Čvek D., Lovrić N., Delaš F. (2010) Aflatoxin M1 in raw milk and binding of aflatoxin by lactic acid bacteria. *Dairy* 60 (4), 244-251

Niderkorn V., Boudra H., Morgavi D. P. (2006) Binding of *Fusarium* mycotoxins by fermentative bacteria *in vitro*. *Applied and Environmental Microbiology* 101, 849–856.

Peltonen K., El-Nezami H., Haskard C., Ahokas J., Salminen S. (2001) Aflatoxin B1 binding by Dairy Strains of Lactic Acid Bacteria and Bifidobacteria. *Journal of Dairy Science* 84, 2152-2156

Pieridis M., El-Nezami H., Peltonen K., Salminen S., Ahokas J. (2000) Ability of dairy strains of lactic acid bacteria to bind aflatoxin M1 in a food model. *Journal of Food Protection* 63, 645-650.

Zinedine A., Soriano J.M., Moltõ J.C., Mañes, J. (2007) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food and Chemical Toxicology* 45, 1-18.