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The Elimination of Ochratoxin A by Lactic Acid Bacteria Strains

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A b s t r a c t

The aim of this study was to examine 29 strains of lactic acid bacteria of the *Lactobacillus* and *Lactococcus* genera, assessing their sensitivity to ochratoxin A and their ability to remove it from a liquid medium. It was demonstrated that most strains are insensitive to the presence of OTA at the quantity of 5 μg/disc. It was demonstrated that all strains caused a reduction of the toxin amount in the liquid medium. The highest decrease, exceeding 50% of the initial OTA content, was caused by the strains *Lactobacillus acidophilus* CH-5, *L. rhamnosus* GG, *L. plantarum* BS, *L. brevis* and *L. sanfranciscensis*. The example of three selected strains confirmed the negative effect of the toxin on the dynamics of bacterial growth. A sharp decrease of ochratoxin A was observed during the first 15 hours of culture growth. In the course of cultivation the amount of the toxin in the medium increased, indicating that the toxin elimination is partially reversible. A small quantity of ochratoxin A became bound to the bacterial biomass.

K e y w o r d s: ochratoxin A, lactic acid bacteria, *Lactobacillus*, mycotoxin elimination

Introduction

Most common contaminating agents of food raw materials and products include fungal metabolites: mycotoxins, and among them ochratoxin A (OTA), which most frequently occurs in the countries of Central Europe, also in Poland (Böhm, 1995). The toxin is produced by numerous fungal species belonging to the genera *Aspergillus* and *Penicillum*, which grow on plant materials stored in excessively humid conditions. It is heat resistant and is not destroyed during thermal processing, also contaminating final food products, *e.g.* bread (Alldrick, 1996). If domestic animals are fed with contaminated feed, ochratoxin A cumulates in their bodies and remains in food products of animal origin (Petzinger and Weidenbach, 2002).

Ochratoxin A owes its toxicity to its chemical structure: a chlorine atom and a phenol group, as well as a molecule of isocumarin play the main role. Ochratoxin A is believed to be carcinogenic, genotoxic, teratogenic, immunosuppressive and nephrotoxic (Petzinger and Ziegler, 2000).

In the case of plant raw materials processed by means of biotechnological tools and used as animal feeds, protection against mycotoxins consists mainly in providing proper cultivation, harvesting and storage conditions (Doyle *et al.*, 1982; Northolt and Bullerman, 1982; Park, 1993). The use of chemical substances and physical processes is allowed to eliminate toxins from animal feeds, a number of requirements must be met, though, *e.g.* the nutritive and sensory value as well as the physical properties of the product have to be maintained, and the decontamination process has to be economically viable (Sinha, 1998).

Detoxification of raw materials used in food processing poses a more serious problem. New opportunities have been created by biological methods, involving the elimination of mycotoxins by microorganisms. Reviews of literature on the subject of biodegradation have been presented by a number of authors (Bhatnagar *et al.*, 1991; Bata and Lasztity, 1999; Karlovsky, 1999). The ability to eliminate ochratoxin A from the environment has been observed for bacteria: *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994), *Phenylobacterium inmobile* (Wegst and Lingens, 1983) and *Saccharomyces cerevisiae* (Štyriak *et al.*, 1998). *Aspergillus fumigatus* and *Aspergillus niger* are also capable of OTA degradation (Varga *et al.*, 2000). Enzymatic hydrolysis of the peptide bond, resulting in formation of ochratoxin a and release of

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phenyloalanine, is considered in literature as the basic toxicity reduction mechanism for ochratoxin A. Peptide bond hydrolysis is carried out by rumen microflora (Hult *et al.*, 1976; Özpinar *et al.*, 1999) and, to a lesser degree, in the alimentary tract of monogastric animals (Madhyastha *et al.*, 1992; Li *et al.*, 2000). Of all microorganisms capable of OTA elimination, lactic acid bacteria attract greatest interest. They have a positive effect on human health, they are safe, and they are applied as a biological agent in numerous biotechnological processes involving raw materials potentially contaminated with ochratoxin A (Wood, 1998). Lactic acid bacteria *Lactococcus salivarius, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Bifidobacterium bifidum* cause a decrease in OTA level in milk (Škinjar *et al.*, 1996).

In the presented study, lactic acid bacteria strains of intestinal and plant origin were assessed for their sensitivity to the presence of ochratoxin A in the medium and their ability to eliminate ochratoxin A.

The process of OTA elimination from the liquid culture medium during the subsequent phases of growth has been described on the example of the most effective strains.

Experimental

Materials and Methods

Bacterial strains. 29 strains of lactic acid bacteria were used in the research: *Lactobacillus rhamnosus* GG ATCC 53105 received from Collegium Medicum, Jagiellonian University, Cracow, *Lactobacillus acidophilus* CH-5 and A92 from Prague Technical University, Department of Milk and Fat Technology, from Danisco Biolacta, Olsztyn: *Lactobacillus acidophilus* CH-2, H-1 and lnd 1, *Lactobacillus delbrueckii subsp. bulgaricus* strains J7, P7, 171/2, SL, *Lactobacillus helveticus* strains 8, D(a), 3035, CH-1, E8, *Lactobacillus casei* B, 150, 18H, 18cz strains, *Lactococcus lactis subsp. lactis* 168, Cz, 147, 8FD, 202, from National Collection of Agricultural and Industrial Microorganisms, Budapest: *Lactobacillus plantarum* strains B1074, B1149, from Collection of Industrial Microorganisms of Institute of Technology Fermentation and Microbiology ŁOCK 105: *Lactobacillus plantarum* ŁOCK 0862, *Lactobacillus sanfranciscensis* ŁOCK 0867 and *Lactobacillus brevis* ŁOCK 0845

The biomass was stored in 15% glycerol at –20°C. Before each experiment, a portion of bacterial biomass was defrosted and activated by a single passage in liquid MRS medium (MERCK) and cultivation for 24 hours at 37°C.

Ochratoxin A standard. Ochratoxin A standard (SIGMA-Aldrich, St Louis, MO, USA) was stored as stock solution in absolute ethanol (200 ppm) at –20°C.

Determination of sensitivity of lactic acid bacteria to the presence of ochratoxin A. The degree of sensitivity of the strains of lactic fermentation bacteria to the presence of ochratoxin A was assayed by disc diffusion method (Xiao *et al.*, 1996). A suspension of the bacteria was added to MRS agar medium, in the quantity sufficient to obtain the final number of cells amounting to *ca.*10^6 cells in 1 cm^3 of the medium. After thoroughly mixing the medium, it was poured into sterile Petri dishes, 15 cm^3 into each dish. Sterile paper discs, 10 mm in diameter, soaked with solutions prepared from standard ochratoxin A solution at the quantity of 20 µl were placed on the surface of the medium. The content of ochratoxin A on the disc amounted to 0.1; 0.5; 1, 5 and 10 µg. After 48 hours of incubation at the temperature of 37ºC it was examined if zones inhibiting the growth of the investigated microorganisms appeared around the discs with specific OTA concentrations or not. The strains' viability was also controlled by plating them on MRS medium.

Screening of the strains capable to reduce the amount of ochratoxin A in model media. Liquid MRS medium (Merck) was used in the studies at the quantity of 10 cm^3 supplemented with 0.05 cm^3 of ochratoxin A standard solution. The initial concentration of ochratoxin A in the medium was 1000 ppb. The media were inoculated with biomass suspended in physiological salt solution (5% vol.) derived from the logarithmic growth phase with density of 10^7 CFU/cm^3. The bacterial cultures were conducted in static conditions at the temperature of 37ºC, for 120 hours, then the cultures were centrifuged (3500 rpm, 10 min) and the amount of ochratoxin A was determined in the post-culture liquid.

The decrease of the toxin in the medium in relation to the initial value was expressed in percentages.

Assessment of changes in the amount of ochratoxin A during cultivation. Ochratoxin A standard solution was added to 50 ml of liquid MRS medium. The initial concentration of OTA was *ca.* 1000 ppb, and was precisely determined at time t = 0. Then inoculum was added, consisting of the tested microorganisms in the exponential growth phase, suspended in normal saline (10^7 cells ml^{-1}). The culture was grown at 37ºC.

The amount of ochratoxin A in the post-culture liquid and the biomass was determined after 5, 15, 24 and 40 hours of cultivation. The number of bacterial cells was calculated with standard plate method on MRS medium at the same time intervals.

Ochratoxin A analysis. Immunoenzymatic quantitative method ELISA was applied to determine the concentration of ochratoxin A in the biomass and in the post-culture medium, after the biomass was centrifuged. The test used was Ridascreen®Ochratoxin A made by R-Biopharm, Darmstadt, Germany. The extraction procedure was applied in accordance with the producer's instructions. The detection limit of this test is 80 ppt.

Statistical Analysis. The results presented in this study are the average of three measurements. Variance analysis (one-way ANOVA test) was performed with the Microcal (TM) ORIGIN ver. 6.0 software (Northampton, USA).

Results

The first stage of the study concerned a comparison of the sensitivity of lactic acid bacteria to the action of different concentrations of ochratoxin A. It was discovered that only one of all examined strains – *Lactobacillus helveticus* CH-1 was sensitive to ochratoxin A in the whole studied range of concentrations, *i.e.* from

Table I
The sensitivity of lactic acid bacteria to the presence of ochratoxin A

Species	Strain	µg ochratoxin A/disc				
		0.1	0.5	1	5	10
Lactobacillus acidophilus	CH-2	–	–	–	–	+
	A92	–	–	–	–	+
	H-1	–	–	–	+	+
	Ind 1	–	–	–	+	+
	CH-5	–	–	–	–	–
Lactobacillus delbrueckii subsp. *bulgaricus*	J7	–	–	–	–	+
	P7	–	–	–	–	+
	171_2	–	–	–	–	+
	SL	–	–	–	–	+
Lactobacillus helveticus	8	–	–	–	–	+
	D(a)	–	–	–	–	+
	CH-1	+	+	+	+	+
	3035	–	–	–	–	+
	E 8	–	–	–	–	+
Lactobacillus casei	B	–	–	–	–	+
	150	–	–	–	–	+
	18H	–	–	–	–	+
	18 cz	–	–	–	–	+
Lactococcus lactis, subsp. *lactis*	168	–	–	–	–	+
	CZ	–	–	–	–	+
	147	–	–	–	–	+
	8FD	–	–	–	–	+
	202	–	–	–	–	+
Lactobacillus plantarum	B 1074	–	–	–	–	+
	B 1149	–	–	–	–	+
	BS	–	–	–	–	+
Lactobacillus sanfranciscensis	BS	–	–	–	–	+
Lactobacillus brevis	BS	–	–	–	–	+
Lactobacillus rhamnosus	GG	–	–	–	–	–

(+) the presence of inhibition zone, sensitive strain

0.1 µg to 10 µg (Table I). The remaining strains showed no sensitivity to this toxin in concentrations ranging from 0.1 to 5 µg, with the exception of the strains *Lactobacillus acidophilus* Ind1 and *L. acidophilus* H-1, whose growth was inhibited by OTA at the quantity of 5 µg. The highest dose of the mycotoxin used in the experiment, *i.e.* 10 µg proved to be inhibitory for the growth of most strains with the exception of *L. acidophilus* B, *L. acidophilus* CH-5 and *Lactobacillus rhamnosus* GG. Strains with the highest sensitivity to ochratoxin A, *i.e. L. acidophilus A92*, *L. acidophilus* Ind1 and *L. helveticus* CH-1 were excluded from further tests.

The next experiment aimed to demonstrate whether the phenomenon of ochratoxin A elimination by lactic acid bacteria exists and which of the investigated microorganisms exhibits this feature most strongly. It was proved that the ability to reduce the amount of ochratoxin A is common among lactic acid bacteria but it is varied, depending on the species and the strain of bacteria.

Taking into account the average values for the species, it is possible to distinguish those with the greatest ability to remove the toxin: *Lactobacillus acidophilus*, *L. rhamnosus*, *L. sanfranciscensis* and *L. plantarum* (Figure 1). The remaining species were characterized by removing the toxin in a much smaller quantity. However, the analysis of the values obtained for particular strains within species revealed considerable differentiation, indicating that the examined feature is strain-specific. Out of the 25 investigated strains 10 were capable of removing about a half of the amount of ochratoxin A from the liquid medium. The biggest decrease of the toxin was observed in the culture of intestinal lactobacilli *L. acidophilus* CH-5, *Lactobacillus*

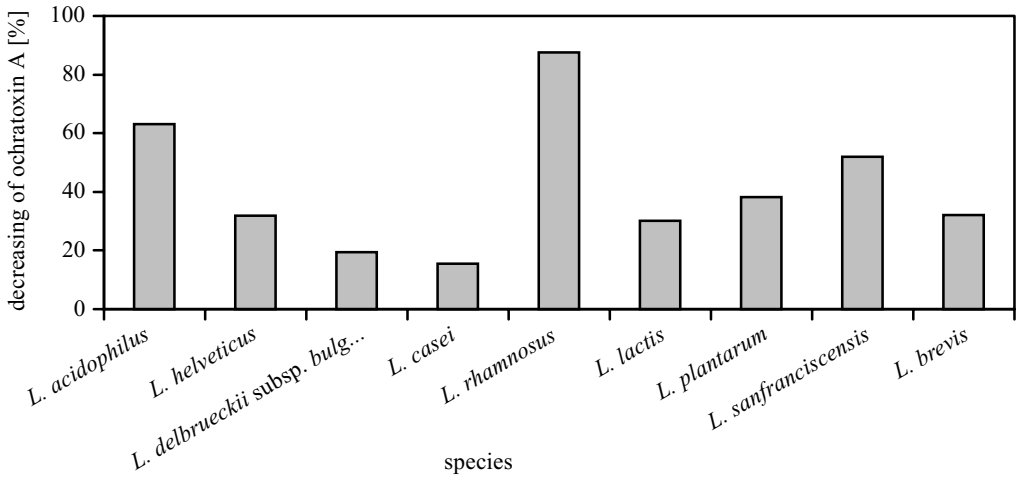


Fig. 1. The elimination of ochratoxin A by different species of lactic acid bacteria

rhamnosus GG, 70.5% and 87.5% respectively (Table II). These strains were also least sensitive to the presence of ochratoxin A in the environment.

A somewhat smaller decrease, about 50% reduction of the OTA amount, was observed in the strains of lactic acid bacteria of plant origin *Lactobacillus plantarum*, *L. sanfranciscensis* and *L. brevis.* These strains were classified into a group of organisms moderately resistant to ochratoxin A (Table I). A small degree of OTA elimination from the medium, amounting to approximately 10%, was demonstrated for 7 strains belonging to different species with the same sensitivity to the toxin as the ones mentioned earlier. The comparison of these data leads to the conclusion that there is no correlation between the sensitivity of the strains to the toxin and the ability to eliminate it.

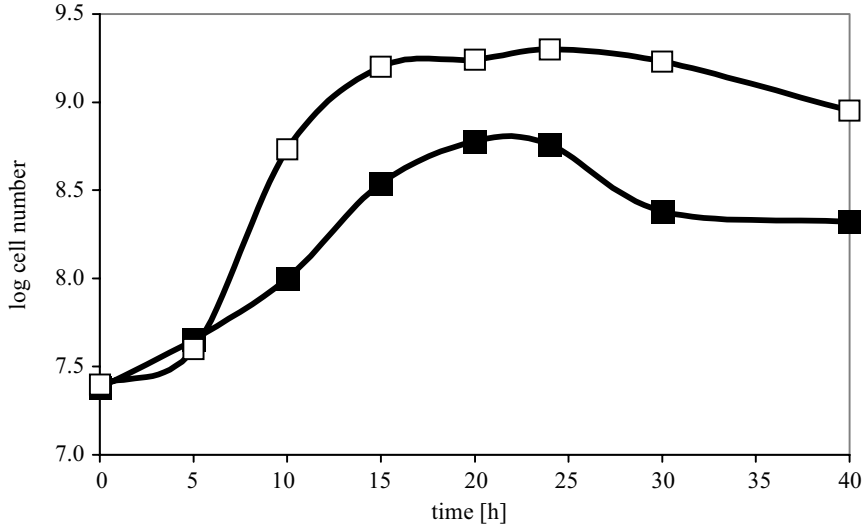
The first stage of the study aimed to demonstrate whether the phenomenon of ochratoxin A elimination by lactic acid bacteria exists and which of the investigated microorganisms exhibits this feature most strongly. It was proved that the ability to reduce the amount of ochratoxin A is common among lactic acid bacteria but it is varied, depending on the species and the strain of bacteria.

Three strains – *L. acidophilus* CH-5, *L. rhamnosus* GG and *L. plantarum* ŁOCK 0862 – were selected for further tests, the purpose of which was to evaluate the impact of ochratoxin A on the increase of bacterial biomass and to observe changes in the amount of ochratoxin A during cultivation on liquid medium. The presence of 1000 ppm of ochratoxin A in the medium slowed down the multiplication rate of the lactic

Table II
The decrease of the amount of ochratoxin A in model medium

Species	Strain	Decreasing of ochratoxin A [%]
Lactobacillus acidophilus	CH-2	45.1 ± 0.72
	A92	50.2 ± 0.62
	CH-5	70.5 ± 0.98
Lactobacillus delbrueckii subsp. *bulgaricus*	J7	5.9 ± 0.62
	P7	34.3 ± 0.46
	171 $_2$	9.6 ± 0.26
	SL	28.3 ± 0.53
Lactobacillus helveticus	8	67.1 ± 0.46
	D(a)	11.9 ± 0.44
	3035	17.0 ± 0.26
	E 8	31.0 ± 0.36
Lactobacillus casei	B	11.5 ± 0.44
	150	29.8 ± 0.30
	18H	5.7 ± 0.44
	18 cz	16.6 ± 0.46

Species	Strain	Decreasing of ochratoxin A [%]
Lactococcus lactis	168	16.8 ± 0.62
	CZ	45.7 ± 0.26
	147	21.0 ± 0.53
	8FD	7.8 ± 0.61
	202	59.6 ± 0.44
Lactobacillus plantarum	B 1074	11.9 ± 0.75
	B 1149	35.5 ± 0.46
	BS	56.2 ± 0.72
Lactobacillus sanfranciscensis	BS	52.0 ± 0.53
Lactobacillus brevis	BS	56.2 ± 0.72
Lactobacillus rhamnosus	GG	87.5 ± 0.66



Fig, 2. The effect of ochratoxin A on biomass yield of *Lactobacillus acidophilus* CH-5, (□), toxin-free medium; (■), medium with toxin

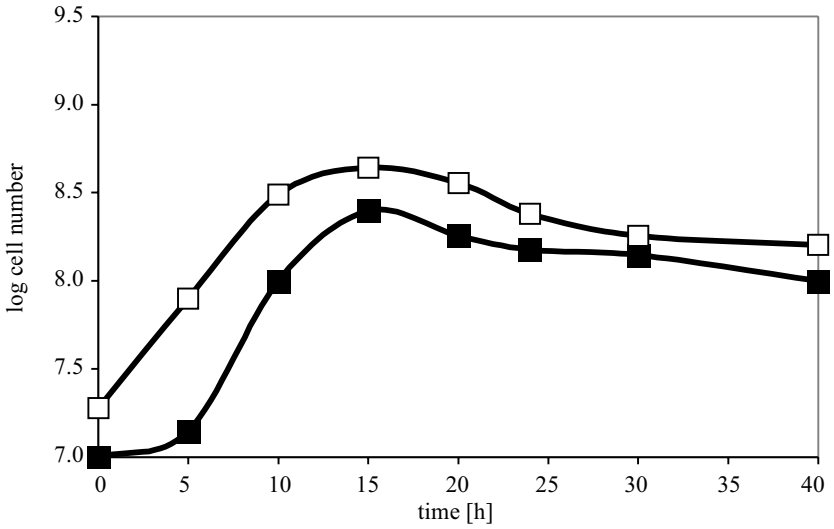


Fig. 3. The effect of ochratoxin A on biomass yield of *Lactobacillus rhamnosus* GG, (□), toxin-free medium; (■), medium with toxin

acid bacteria (Figures 2, 3). Maximum biomass yield for *L. acidophilus* CH-5 (6.0×10^8 CFU ml^{-1}) was only reached after 20 hours, for the control – 5 hours earlier. The cell number was over three times higher in the toxin-free medium, amounting to 1.9×10^9 CFU ml^{-1} (9.3 Log value). The other strain, *L. rhamnosus* GG, proved to be less sensitive to the presence of ochratoxin A in the medium. Maximum yield in both toxin-free and contaminated medium was reached at the same time, *i.e.* after 15 hours of growth. The cell number in the medium with OTA was 2.0×10^8 CFU ml^{-1}, only twice lower than in control (Figure 3). A similar negative effect of ochratoxin A on the increase of bacterial biomass was observed for *L. platarum*.

The changes in the content of ochratoxin A in the post-culture medium are presented in Figure 4. The amount of OTA in the *L. acidophilus* CH-5 culture plunged dramatically during the first 5 hours, reaching the level of 145 ppb, which means that 85% of the initial OTA content was eliminated. After the next 10 hours, the amount of toxin dropped to 120 ppb. Starting from the 15th hour of incubation, the release of toxin to the medium restarted and lasted until the 40th hour of the process. The level of toxin in the medium finally reached 270 ppb, which means that 17% of the previously eliminated toxin (150 ppb) returned into the medium during the entire growth process. The difference in the OTA level between the 15th and 40th hour is of statistical relevance ($P<0.05$). During the 40-hour growth of *Lactobacillus acidophilus* CH-5, ochratoxin A was eliminated in 72% (Figure 4). For the strain of *Lactobacillus rhamnosus* GG, a similar process of ochratoxin A reduction was observed. However, the first 5 hours were not as effective as in the

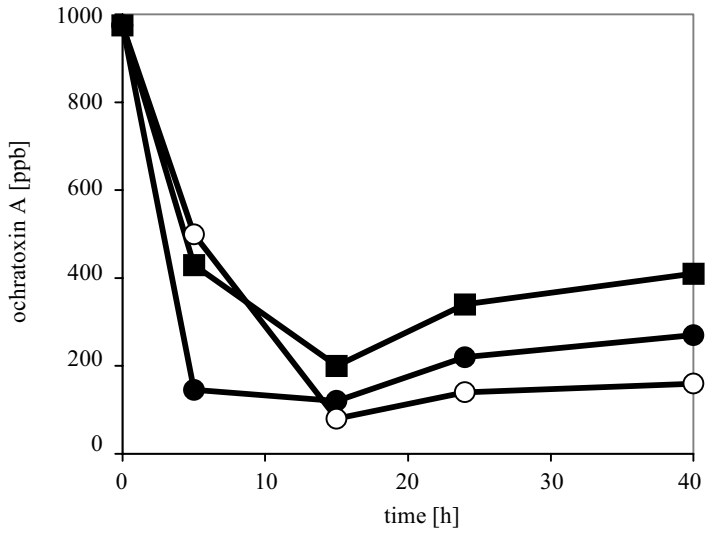


Fig. 4. Change dynamics of the amount of ochratoxin A in the cultures of lactic acid bacteria, (○) , *Lactobacillus rhamnosus* GG; (●), *L. acidophilus* CH-5; (■), *Lactobacillus plantarum* ŁOCK 0862

case of the other strain, as only 50% of the initial toxin amount was eliminated. The lowest OTA concentration in the post-culture medium, 80 ppb, was observed during the 15th hour of growth, which means that 90% of toxin had been removed. After 15 hours of incubation, an increase ($P<0.05$) in the toxin level occurred, and it reached 160 ppb in the 40th hour. Thus, between the 15th and 40th hour of incubation, about 9% of previously eliminated toxin returned into the medium.

During cultivation of the strain *Lactobacillus plantarum* BS the amount of ochratoxin A also dropped significantly in the medium in the first 5 hours of incubation, reaching 30 ppm. The next stages of cultivation, *i.e.* to the 15th hour resulted in a further decrease to the level of 200 ppm. Just as it occurred in the cultures of previously discussed strains, after the 15th hour of incubation a release of a part of OTA to the medium was observed. Between the 15th and the 24th hour of incubation 140 ppm returned to the environment, *i.e.* 17.5% of the previously eliminated toxin, prolonging the cultivation to 40 hours resulted in an increase of the amount of the toxin in the medium to 410 ppm, which is a level comparable to that obtained after 5 hours of cultivation. In the *L. plantarum* culture the amount of the toxin that returned to the medium was bigger than for the other strains.

According to literature data concerning aflatoxin B_1 (El-Nezami *et al.*, 1998), elimination of the toxin from the environment occurs through binding it to the bacterial biomass. To find out if this hypothesis is also correct for the examined toxin, the amount of OTA in the centrifuged bacterial biomass was determined (Table III). The values in the table are expressed in pg, they take account of the level of cell multiplication and relate to one colony forming unit (CFU). The content of ochratoxin A in the biomass extract of *L. acidophilus* CH-5 increased in the course of growth, reaching 0.167 mg in the 15th hour of incubation, which means that 0.00012 pg of toxin was bound by each CFU. The prolongation of the culture up to 40 hours resulted in a further increase in the amount of OTA up to 0.232 mg, *i.e.* 0.0011 pg CFU^{-1}. In the case of the other strain, *L. rhamnosus* GG, a similar tendency was observed, but the amounts of toxin bound by the biomass were higher: 0.00023 pg CFU^{-1} in the 15th and 0.0021 pg CFU^{-1} in the 40th hour of growth. Upon analysis of the results presented in Table III, it can be assumed that ochratoxin A is bound by the cells of lactic acid bacteria. However, it is impossible to balance the amount of ochratoxin A, which suggests that apart from binding to the biomass there is another mechanism of removing ochratoxin A.

Table III
Ochratoxin A in bacterial biomass

Time [h]	Ochratoxin A [pg/CFU]	
	Lactobacillus acidophilus CH-5	*Lactobacillus rhamnosus* GG
15	0.00012	0.00023
24	0.00047	0.00082
40	0.0011	0.0021

Discussion

The scarce published studies suggest that bacteria, including lactic acid bacteria, are microorganisms resistant to this toxin, although the toxicity thresholds reported by different authors are varied. According to the studies of Xiao *et al.* (1996) on biological activity of OTA in relation to *Bacillus brevis*, 2 μg/disc is the lowest dose of the toxin able to create a zone inhibiting growth, which is less than in the case of the strains of lactic fermentation bacteria investigated in this study.

The above-presented values demonstrate that ochratoxin A has a negative effect on the growth of lactic acid bacteria. The results are different from those presented by Ali-Vehmas *et al.* (1998), which showed that ochratoxin A at 20 ppm does not inhibit the growth of *L. plantarum* and *L. casei*.

The results of this study demonstrate that the tested strains of lactic acid bacteria, *L. acidophilus* CH-5, the probiotic *L. rhamnosus* GG and lactobacilli of plant origin *L. plantarum* are capable of eliminating ochratoxin A from model media. The process is partly reversible and, upon culture prolongation, the part of toxin is released back into the medium after 40 hours of incubation. A part of the toxin becomes bound by the bacterial biomass, however, the remaining amount is eliminated in a different way. These results are consistent with the studies on aflatoxin B_1, according to which the strain of *L. rhamnosus* GG is capable of eliminating 80% of toxin from the medium exclusively through physical binding by the cell wall components (El-Nezami *et al.*, 1996; 1998; 2000).

On the basis of the conducted studies it is possible to conclude that the application of selected strains of lactic acid bacteria in the production of fermented food and probiotic products may reduce the health risk related to the possible contamination of food products exposition to fungal toxins. These strains may protect directly humans eating several foods contaminated by toxins too.

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