Microbiologicals for deactivating mycotoxins

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Mycotoxins are secondary metabolites of fungi affecting human and animal health. Five classes of mycotoxins are of major concern in animal husbandry, namely aflatoxins, trichothecenes, zearalenone, ochratoxins, and fumonisins. Due to their diverse structure these fungal toxins are able to cause a great variety of acute symptoms in animals. Clay minerals have been used in animal nutrition to bind mycotoxins, but the binders are only very specific for aflatoxins but not for other toxins. A novel strategy to control the problem of mycotoxicoses in animals is the application of microorganisms capable of biotransforming mycotoxins into nontoxic metabolites. The microbes act in the intestinal tract of animals prior to the resorption of the mycotoxins. A Eubacterium (BBSH 797) strain is able to deactivate trichothecenes by reduction of the epoxide ring (CAST, *Mycotoxins, Risks in Plant, Animal and Human Systems, Task Force Report 139*, Council of Agricultural Science and Technology, Ames Iowa 2003, p. 10.; Binder, E. M., Binder, J., Ellend, N., Schaffer, E. *et al.*, in: Miraglia, M., van Egmond, H., Brera, C., Gilbert, J. (Eds.), *Mycotoxins and Phycotoxins – Developments in Chemistry, Toxicology and Food Safety*, Alaken, Fort Collins 1996, pp. 279 – 285). This strain was isolated out of bovine rumen fluid and the mode of action was proven in vitro and also in vivo. Further a novel yeast strain, capable of degrading ochratoxin A and zearalenone was isolated and characterized (Bruinink, A., Rasonyi, T., Sidler, C., *Nat. Toxins* 1999, *6*, 173 – 177; Schatzmayr, G., Heidler, D., Fuchs, E., Mohnl, M. *et al.*, *Mycotoxin Res.* 2003, *19*, 124 – 128.) Due to the yeasts affiliation to the genus of Trichosporon and its property to degrade mycotoxins this strain was named *Trichosporon mycotoxinivorans* (Trichosporon MTV, 115).

Keywords: BBSH / Biotransformation / Mycotoxins / Trichosporon mycotoxinivorans / Trichothecenes

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1 Introduction

1.1 General remarks

Mycotoxins are highly toxic secondary metabolic products of moulds mainly belonging to *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* species. Under certain conditions they produce mycotoxins, with the group of trichothecenes (*e. g.*, deoxynivalenol, T-2 toxin), zearalenone, ochratoxins, aflatoxins, and fumonisins being the most prevalent. These toxins cause substantial economic losses in animal husbandry.

The global occurrence of mycotoxins is considered to be a major risk factor, as according to the Food and Agricultural Organization (FAO) 25% of the world's commodities are annually affected by known mycotoxins. In the United States only, the mean economic annual costs of crop losses caused by mycotoxins are estimated to be 932 million USD. [1]. Contamination of food and feed occurs in the field before harvest or during storage despite the most strenuous efforts of prevention [2]. Therefore practical detoxification procedures for feed have been developed. These detoxification strategies can be grouped into three categories: physical, chemical, and biological methods [3]. Methods, investigated for physical detoxification, are cleaning, mechanical sorting and separation, washing, density segregation, ther-

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Abbreviations: AMM, anaerobic minimal medium; **LPA,** lymphocyte proliferation; **MAT,** macrophage activation test; **MM,** minimal medium; **NO,** nitric oxide; **OTα**, ochratoxin alpha; **rpm,** revolutions *per* minute

mal inactivation, irradiation (including microwaves), ultrasound, and solvent extraction. In the past also chemicals, like ammonium hydroxide to detoxify deoxynivalenol or calcium hydroxide monomethylamine to diminish the content of aflatoxin, T-Toxin, HT-2 toxin, deoxynivalenol, and zearalenone, have been tested. However, these physical and chemical detoxification methods often do not work, are too costly and on the other hand, often destroy or remove essential nutrients from the feedstuff and reduce palatability [4]. Biological detoxification is the method of choice to deactivate mycotoxins. This comprises binding by adsorptive materials as well as microbial inactivation by specific microorganisms or enzymes. Up to now a lot of research has been done to adsorb or deactivate mycotoxins in feed with products that can be mixed into the feed. While the use of products based on aluminosilicates gave good results in counteracting aflatoxins [5, 6], the adsorptive deactivation of other toxins failed under field conditions [7–10]. Especially hydrated sodium alumino silicates (HSCAS) have been extensively studied because of their promising aflatoxin-binding capacity. Although HSCAS were demonstrated to be very effective with regard to preventing aflatoxicosis, their efficacy against zearalenone and ochratoxin A is limited and in the case of trichothecenes, it is practically zero [11].

An efficient way of detoxification of mycotoxins that can poorly be bound is microbial or enzymatic detoxification. This approach is defined as biodegradation or biotransformation that leads to less toxic products. Research in the field of mycotoxin detoxification by microorganism is not a new issue – reports on this subject have been available in the past three decades. A few microorganisms with mycotoxin degradation activity were isolated, the first was *Flavobacterium aurantiacum* with the ability to detoxify aflatoxins [12]. Wegst and Lingens [13] proved degradation of ochratoxin A by the aerobic bacterium *Phenylobacterium immobile*. *Gliocladium roseum* detoxified zearalenone by ring opening with subsequent decarboxylation in yields ranging between 80 and 90% [14].

In the case of trichothecenes it is known, that the 12,13-epoxide ring is responsible for their toxic activity and removal of this epoxide group entails a significant loss of toxicity. Several authors described this de-epoxidation reaction of ruminal or intestinal flora [15–18], but Binder *et al.* [19] were the first to isolate a pure bacterial strain, which was able to biotransform the epoxide group of trichothecenes into a diene [20]. Deoxynivalenol, the best known trichothecene is enzymatically reduced by an epoxidase of *Eubacterium* BBSH 797 to the nontoxic metabolite de-epoxy-deoxynivalenol (DOM-1). This strain was isolated out of bovine rumen fluid. The mode of action was proven *in vitro* and also *in vivo* by applying trichothecenes, and the detoxifying strain *Eubacterium* BBSH 797 has been the first microbe used in a mycotoxin deactivating in feed additive [21].

Further research was related to ochratoxin A and zearalenone detoxification, because binding materials are known to have a poor ability to adsorb these compounds. Recently, a novel yeast strain was isolated and characterized, which has the capability of degrading ochratoxin A and zearalenone. Due to the yeast affiliation to the genus *Trichosporon* and to its main property to degrade OTA and ZON (*lat. vorare* = degrade), this strain was named *Trichosporon mycotoxinivorans* (*MTV, 115*) [22]. The yeast can detoxify OTA by cleavage of the phenylalanine moiety from the isocumarin derivate ochratoxin alpha (OTα). This metabolite has been described to be nontoxic or at least 500 times less toxic than the parent compound [23, 24]. Zearalenone has no acute toxicity, but it mimics the reproduction hormone estrogen, and therefore causes substantial fertility problems. The metabolization of zearalenone by *T. myctoxinivorans* leads to a compound that is no longer estrogenic. This has been proven in an *in vitro* assay with breast cancer cells [24].

1.2 Objectives

Due to the limitation of available methods to detoxify certain mycotoxins in animal feeds a screening for OTA-degrading microorganisms was carried out. Isolates capable of cleaving OTA were compared with each other and also with already described microorganisms for their suitability to be used in an animal feed additive to counteract this mycotoxin. The best strain resulting from this screening should be tested in an *in vitro* model based on pig intestine. Furthermore, a bacterium capable of degrading trichothecenes [20] should be tested in this model for its ability to degrade DON in a complex matrix. Finally the degradation products should be evaluated in a lymphocyte proliferation (LPA) assay (in case of DON) and a macrophage activation test (MAT) (in case of OTA).

2 Materials and methods

2.1 Mycotoxins

Ochratoxin A was produced by *Aspergillus ochraceus* on cracked wheat (4 wk at 30°C) and extracted with chloroform. Then this solvent was evaporated and the residue dissolved in Tris-HCl. Zearalenone was purchased from Sigma. For the use in biotransformation experiments with *Eubacterium BBSH 797*, DON and 3-AcDON were prepared from *Fusarium graminearum* cultures (DSMZ 4258) following the method of Altpeter and Posselt [25].

2.2 Enrichment and degradation studies

Segments of intestines from freshly slaughtered pigs were put in a sealed glass bottle and immediately transported to the laboratory. Rumen fluid, obtained from a cannulated cow, was placed in a glass container, flushed with nitrogen, and taken to the laboratory. In screening experiments for anaerobic bacteria from rumen fluid and intestinal contents a growth medium (MM10) for cultivation and maintenance of anaerobic bacteria was used [26]. For enrichment of aerobic bacteria nutrient broth (Merck, Darmstadt) was used. For cultivation of yeast strains a culture medium (YM) consisting of malt extract (10 g/L), glucose (10 g/L), yeast extract (5 g/L), and peptone (5 g/L) was employed. The degradation activity of anaerobic bacteria was also tested in an anaerobic minimal medium (AMM) with the following composition (in 1 L of distilled water): 75 mL of mineral solution I (6 g/L K_2HPO_4); 75 mL of mineral solution II (6 g/L KH_2PO_4, 6 g/L $(NH_4)_2SO_4$, 12 g/L NaCl, 2.5 g/L $MgSO_4 \times 7H_2O$, 3 g/L $CaCl_2 \times 2H_2O$); 10 mL of vitamin solution (2 mg/L biotin, 2 mg/L folic acid, 10 mg/L pyridoxine × HCl, 5 mg/L thiamine × HCl, 5 mg/L riboflavin, 5 mg/L nicotinic acid, 5 mg/L DL-Ca-pantothenate, 0.1 mg/L cyanocobalamin, 100 mg/L menadione, 22 mg/L K_1, 1, 5 mg/L *p*-aminobenzoic acid, 5 mg/L thioctic acid); 0.5 mL of trace element solution I (0.10 g/L $ZnSO_4 \times 7H_2O$, 0.03 g/L $MnCl_2 \times 7H_2O$, 0.30 g/L H_3BO_4, 0.01 g/L $CuCl_2 \times 2H_2O$, 0.20 g/L $CoCl_2 \times 6H_2O$, 0.02 g/L $NiCl_2 \times 6H_2O$, 0.03 g/L $Na_2MoO_4 \times 2H_2O$); 0.5 g cysteine × HCl, pH 6.8 – 6.9. Anaerobic cultures were incubated at 37°C for 3 – 5 days. The headspace of each bottle was flushed with nitrogen, carbon dioxide, or hydrogen to obtain an anaerobic atmosphere.

The mycotoxin degrading activity of aerobic strains (aerobic bacteria and yeast) was investigated in the respective growth medium and in a minimal medium (MM) with the following composition (in 1 L of distilled water): 2.44 g Na_2HPO_4, 1.52 g KH_2PO_4, 0.50 g $(NH_4)_2SO_4$, 0.20 g $MgSO_4 \times 7H_2O$, 0.05 g $CaCl_2 \times 2H_2O$, 10 mL trace element solution II (0.5 g/L EDTA, 0.2 g/L $FeSO_4 \times 7H_2O$, 0.01 g/L $ZnSO_4 \times 7H_2O$, 0.003 g/L $MnCl_2 \times 7H_2O$, 0.030 g/L H_3BO_3, 0.001 g/L $CuCl_2 \times 2H_2O$, 0.020 g/L $CoCl_2 \times 6H_2O$, 0.002 g/L $NiCl_2 \times 6H_2O$, 0.003 g/L $Na_2MoO_4 \times 2H_2O$), vitamin solution 10 mL, 50 mL tap water, pH 7.00. Aerobic cultures were incubated on a shaker at 20, 25, or 30°C depending on their favorite growth temperatures.

2.3 Analysis of mycotoxins

For determining ochratoxin A and its main metabolite ochratoxin α from matrices like culture medium, AMM, MM, and intestinal contents, 1.5 mL of liquid sample was centrifuged and 1 mL of the supernatant was transferred into a glass tube and acidified with 1 M phosphoric acid. Then OTA and OTα were extracted with 5 mL of chloroform by shaking the closed tube vigorously for 1 min. After separation of the phases 4 mL of the chloroform phase was transferred into another glass tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in ACN : water : acetic acid = 45 : 54 : 1 v/v/v) and analyzed by HPLC-FLD (gradient: A = ACN : water : acetic acid = 45 : 54 : 1 v/v/v, B = water : acetic acid = 99 : 1 v/v; column: HP ODS Hypersil 2.1 mm × 10 mm, 3 µm).

Sample clean up for analysis of deoxynivalenol and its nontoxic metabolite DOM-1 was performed by the use of Mycosep® #225 columns purchased from Romer Labs, MO. One milliliter of the buffer solution containing the toxin and the tested microorganisms was transferred into a glass tube and diluted with 4 mL of ACN. After mixing, the whole liquid was pressed through the column. Two microliters of the supernatant was transferred into a glass tube and dried on the heating block at about 60°C. The residue was dissolved in water : ACN : methanol = 96 : 2 : 2 v/v/v and analyzed according to Binder *et al.* [27].

Sample cleanup for zearalenone analysis was performed by the use of Mycosep® #224 columns purchased from Romer Labs. One microliter of buffer solution containing the toxin and the tested microorganisms was transferred into a glass tube and diluted with 4 mL of ACN. After adding 50 µL of acetic acid and mixing, the whole liquid was pressed through the column. Two microliters of the supernatant was transferred into a glass tube and dried on the heating block at about 60°C. The residue was dissolved in ACN : water = 40 : 60 v/v and assayed by HPLC with fluorescence detection.

2.4 Fermentation and stabilization of mycotoxin deactivating microbes

For breeding of pure cultures of mycotoxin deactivating microorganisms a fermenter with a working volume of 5 L (Braun Biostat ED, Biotech International) was used. Cells were concentrated by centrifugation (Beckman J2-MI Centrifuge, 25 min, 9500 revolutions *per* minute (rpm) and dried by a freeze dryer (Christ Beta 1 – 16). For determination of mycotoxin degrading activities 20 mL of fermentation broth was concentrated by Beckmann GS-6 Centrifuge (3900 rpm; 25 min). Afterwards the pellet was suspended in 20 mL, mycotoxin – containing solution (AMM or MM) and incubated for a certain period at the respective temperature (species dependent). The medium was spiked with 1 µg/mL deoxynivalenol, 200 ng/mL ochratoxin A or 1 µg/mL zearalenone. During this incubation time samples were taken at 2.5 or 5 h intervals and mycotoxins were assayed by HPLC as described above.

For investigation of stabilized cells 2 g of the lyophilisate was suspended in 18 mL of the respective medium (AMM or AM), which contained one of the tested mycotoxins (1 μg/mL DON, 200 ng/mL OTA, or 1 μg/mL ZON) and dilution series were made. Each dilution step was analyzed for activity. Therefore dilution steps 10^{-2}, 10^{-3}, and 10^{-4} were incubated at the respective temperatures and analyzed for the respective mycotoxins and their metabolites.

2.5 Test model using gut pieces

In the course of these experiments, whole pieces of gut were used. Pig intestine was taken immediately after slaughter and transported to the laboratory under anaerobic conditions (sealed container under CO_2 – atmosphere). Pieces of defined length (approximately 15 cm) were cut from different gut sections (duodenum, jejunum, and ileum), tied up at the ends, and weighed. The gut pieces were inoculated with defined concentrations of just fermented or already freeze-dried microbial cells and a certain amount of the respective mycotoxin solution, and incubated in a 250 mL glass bottle at 37°C under CO_2 gas atmosphere. Samples for analytical determination of mycotoxins and their metabolites, respectively, were taken periodically (three to four times) within a 24–48 h incubation period. Control batches were prepared without addition of the active strains, in order to assess the degradation activity of the original gut microbes and therefore to avoid false positive results. Within 48 h accompanying biotransformation analyses were carried out periodically (three to four times).

2.6 Sequencing with 16S rDNA

Amplification of the 16S rDNA was performed with a TPersonal Cycler (Biometra, Göttingen, Germany) using the eubacterial primer set 27f and 1492r [28] corresponding to *Escherichia coli* 16S rRNA positions 8–27 and 1492–1513, respectively. A standard 50 μL reaction mixture contained 0.2 mM of dATP, dCTP, dGTP, dTTP (Boehringer Mannheim), 1 unit Dynazyme Polymerase (Finnzymes), 1 × standard PCR-buffer (Finnzymes), autoclaved distilled water, 16S-primers (1 μmol l-1), and template DNA. The PCR program started with an initial denaturing step at 95°C for 5 min, which was followed by a hot start and 30 cycles of DNA denaturation at 94°C for 2 min, primer annealing at 50°C for 1.5 min, and DNA extension at 72°C for 5 min. Amplification products were purified with the QIAquick PCR Purification Kit (Qiagen). Purified PCR products were directly sequenced at the Service Department at the Vienna Biocenter (VBC-GENOMICS Bioscience Research, Vienna, Austria). 16S rRNA gene sequence similarity studies were carried out by using FASTA3.

2.7 Toxicity testing

Detoxification of mycotoxins by the most important isolates was proven by a LPA assay in case of DON and by a MAT for OTA.

For the LPA freshly isolated chicken lymphocytes were cultured in the presence of the respective test substances *in vitro* for a certain period of time, and the amount of proliferated cells was quantified with a colorimetric immunoassay after *in vitro* stimulation. BrdU (5-bromo-2′-deoxyuridine) was added and subsequently incorporated into the DNA of proliferating cells. The amount of incorporated BrdU was measured by an ELISA-method. The developed color and thereby the absorbance values directly correlated to the amount of DNA synthesis and hereby to the number of proliferating cells. At a certain concentration DON inhibited the proliferation of lymphocytes.

The MAT was used to show detoxification of ochratoxin A by a yeast strain. The test was performed with a chicken macrophage cell line (HD-11). Growing macrophages are producing nitric oxide (NO), which is not stable in aqueous solutions and decomposes into nitrate and nitrite, which can be detected with the help of a dye reagent in the supernatant fluid of the cultured cells. NO can be used as a quantitative index of macrophage activation. At a certain concentration ochratoxin A has a negative influence on macrophages and that is the reason why it inhibits growth of cells and subsequently the production of NO. LPS from *E. coli* was used as reference substance in this test.

2.8 Statistical studies

Three replicates of each experiment were conducted and data were analyzed by using SPSS (Statistical Software). Degradation rates were calculated in percent based on the OTA concentration used in the experiment, which was either 200 or 400 μg/L.

3 Results and discussion

By applying enrichment methods like variation of culture medium, carrying out dilution series, and isolation procedures using agar plates one bacterium could be isolated from rumen fluid of cattle which had the activity to cleave OTA into ochratoxin α and phenylalanine. A partial analysis of the 16S rDNA gene of the second isolate from rumen fluid revealed that it is related to *Lactobacillus vitulinus* (see Table 1). Although many authors have reported that protozoa are mainly responsible for the detoxification of ochratoxin A in rumen fluid of cattle and sheep present data

Table 1. Taxonomic allocation of anaerobic, OTA-degrading bacterial isolates obtained from cattle rumen and pig intestines

Designation	Origin	Taxonomic allocation based on partial 16s rDNA sequence	% Similarity	bp analyzed
Ru8	Rumen	*L. vitulinus*	99.8	0 – 500
F6	Intestine	*E. ramulus*	95.7	0 – 500
Due2_20	Intestine	*S. pleomorphus*	99.2	0 – 500
Due4_11	Intestine	*E. callanderi*	99.8	0 – 500
Di1_8	Intestine	*E. callanderi*	99.3	0 – 500

show that bacteria play also an important role in degrading ochratoxin A.

The same cultivation methods have been used for a screening of microorganisms in different segments of pig intestine. At the end of enrichment- and isolation-procedures four bacterial strains were obtained (see Table 1). One of these strains (Due2_20) was closely related to *Streptococcus pleomorphus* (similarity: 99.2%), one (Due4_11) belonged most probably to *Eubacterium callanderi* (99.8%). The 16S rDNA gene of the only OTA-degrading strain from the large intestine (Di1_8) had a similarity of 99.3% to the respective gene of *E. callanderi*. Isolate F6, which was taken from intestinal fluid obtained from the small intestine of a pig had the highest similarity to *Eubacterium ramulus* (95.7%) but is probably a strain of a new species. In comparison with the bacterium isolated from rumen fluid, which sometimes lost its OTA-detoxification capability, intestinal strains showed a more rapid deactivation of this mycotoxin. After suspending cells of Due4_11 in AMM with 200 ng/mL Ochratoxin A, 100 percent of this mycotoxin was deactivated within 6 h. Also aerobic bacteria were enriched from soil samples. For this reason soil samples were incubated in a minimal culture medium containing 400 ng/mL OTA to favor growth of OTA-metabolizing strains. Afterwards streak out cultures on agar plates were made and several bacteria capable of transforming OTA to OTα were isolated. Partial sequencing of the 16S rDNA gene revealed that strains with the highest degradation-activities belonged to the genera *Sphingomonas*, *Stenophomonas*, *Rhodococcus*, and *Ralstonia* (see Table 2).

Schatzmayr *et al.* [24] reported that various yeast isolates are able to degrade ochratoxin A by cleavage of the phenylalanine moiety. These strains belonged to the genera *Trichosporon*, *Rhodotorula*, and *Cryptococcus*. In the present study we compared this yeast strains with our isolates regarding their velocity of ochratoxin A-detoxification in respective test media (AMM for anaerobic strains, MM for aerobic bacteria and yeast).

In the comparison study of anaerobic strains it can be seen that *Eubacterium* Due4 _11 was faster in detoxifying OTA than *Eubacterium* F6 (see Fig. 1). After 6 h this strain had

Table 2. Taxonomic allocation of aerobic, OTA-degrading bacterial isolates obtained from soil

Designation	Origin	Taxonomic allocation based on partial 16s rDNA sequence	% Similarity	bp analyzed
033-1	Soil/water	*Sphingomonas paucimobilis*	96.9	0 – 500
034-1	Soil/water	*Sphingomonas asaccharolytica*	97.5	0 – 500
041-9	Soil/water	*Stenotrophomonas nitritreducens*	99.9	350 – 1500
041-10	Soil/water	*Stenotrophomonas sp.*	96.7	350 – 1500
053	–	*Ralstonia eutropha*	100	0 – 500
041 – 8	Soil/water	*Ochrobactrum sp.*		
043-1a	Soil/water	*Stenotrophomonas spec.*	100	0 – 500
055	–	*Rhodococcus erythropolis*	100	0 – 500
075	–	*Agrobacterium sp.*		
038-1	Soil/water	*Ralstonia basilensis*	99.6	0 – 500

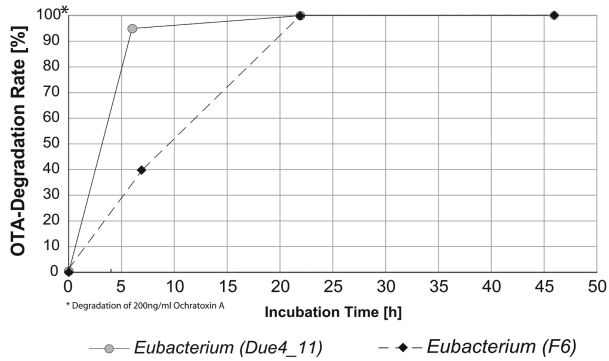

Figure 1. Comparison of anaerobic bacteria isolated from pig intestine concerning deactivation of ochratoxin A.

metabolized 95% of the added OTA (200 µg/L) (see Fig. 1). The highest aerobic activities concerning ochratoxin A-degradation were obtained with *Sphingomonas* (033-1, 034-1) and *Stenotrophomonas* (*e.g.*, 041-9) strains (see Fig.2). Within 5 h of incubation both *Sphingomons* isolates (033-1, 034-1) degraded more than 95% of the added OTA. Isolate 041-9 (*Stenotrophomonas*) needed 10 h for detoxifying 200 µg/L of ochratoxin A. *Ralstonia* and *Rhodococcus* were clearly slower in degrading OTA (degradation rates up to 75% after 55 h) but faster than *P. immobile*. In conclusion isolates from the aerobic bacteria of *Sphingomonas* and *Stenotrophomonas* had the highest activity for decomposition of ochratoxin A.

Degradation rates of tested yeast strains were very similar to that of aerobic bacteria. After 2.5 h *T. mycotoxinivorans* (MTV, 115) had already degraded all ochratoxin A added (see Fig. 3). *Rhodotorula* and *Trichosporon* (178) were slower but had the entire mycotoxin amount degraded after 24 h of incubation. *Cryptococcus* was not able to detoxify the entire OTA within the incubation period of 48 h.

Eight of the best strains were cultivated in a 5 L laboratory fermenter to assess whether stabilized cells (powdered form) could recover in a liquid solution and could deacti-

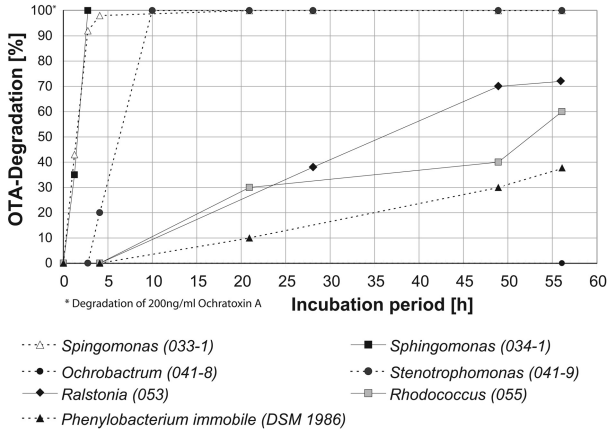

Figure 2. Time course of OTA detoxification by aerobic bacteria isolated from soil.

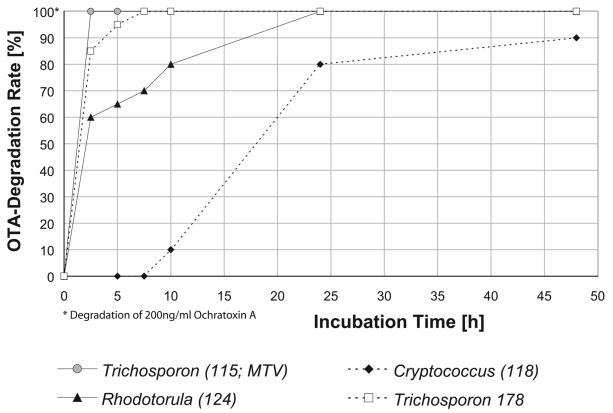

Figure 3. Degradation of ochratoxin A by various yeast strains in dependence of incubation time.

vate ochratoxin A immediately. For the aerobic strains nutrient broth was used as growth medium, temperature was set at 30°C, stirrer speed was adjusted to 200 rpm, and air was used to ensure supply with oxygen. Duration of fermentations was 97 h for strain 033-1, 22 h for strain 041-9, and 70 h for isolate 034-1. After separation of the biomass the concentrate was frozen at −80°C and subsequently freeze-dried. In case of yeasts YM was used to provide all necessary nutrients. Temperature was adjusted to 25°C and the other parameters corresponded with that of the fermentation of aerobic bacteria. The fermentation of *T. mycotoxinivorans* (MTV, 115) was stopped after 22 h and that of *Cryptococcus* 118 after 21 h. The growth of strain 124 and 178 lasted 45.75 and 46.5 h, respectively. The downstream process followed the scheme of fermentation of aerobic bacteria. Production of *Eubacterium* Due4_11 was conducted under anaerobic conditions using MM10 medium and nitrogen for the removal of the oxygen. Temperature was 37°C and stirrer speed 200 rpm. After 48 h the fermentation broth was centrifuged and afterwards lyophilized.

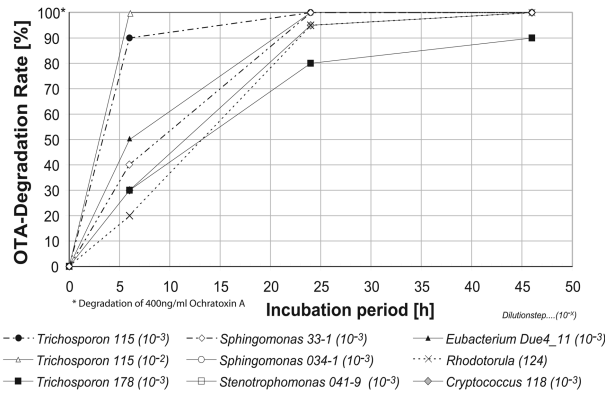

Figure 4. In comparison to Fig. 1, the corresponding OTA-degradation results of respective lyophilisates dissolved in buffer solution. After 5 h (first sampling) *T. mycotoxinivorans* (MTV, 115) and *Stenotrophomonas sp.* degraded between 95 and 100% of OTA (400 ng/mL).

Subsequently each fermentation product was investigated in the MM solution (aerobic bacteria and yeast) and AMM (anaerobic bacterium), respectively. The highest activities concerning ochratoxin A detoxification were obtained by *Stenotrophomonas* 041-9 and *T. mycotoxinivorans* (MTV, 115) (see Fig. 4).

After 6 h 400 ng/mL ochratoxin A was fully decomposed to ochratoxin α. Degradation rates of *Sphingomonas* 034-1 and *Eubacterium* Due4_11 were 50% after 6 h and 100% after 24 h of incubation. The remaining strains could also recover relatively fast but detoxification rates were less than those of the strains mentioned before. Due4_11: With one exception (*Trichosporon* 178) each strain had deactivated all added OTA after 46 h at the latest. All microorganisms investigated in this study were also subjected to a zearalenone degradation study. Cells were grown in the respective medium, centrifuged, and suspended in a MM, which contained 1 μg/mL ZON. During the incubation period samples were taken and analyzed for zearalenone. Only one microorganism (*T. mycotoxinivorans*, MTV, 115) led to a reduction of ZON. In Fig. 5 removal of ZON (retention time 7.45 min) by the yeast is demonstrated. After 24 h at the latest the entire mycotoxin amount was degraded to carbon dioxide or into a non toxic metabolite. α-Zearalenol and β-zearalenol, which are more estrogenic, could not be detected at the end of the degradation study.

3.1 *In vitro* model with pig intestine

A modified *in vitro* test model was developed to prove the detoxification potential of *Eubacterium BBSH 797* [21] under real conditions and also to select the best strain for detoxification of OTA. For the latter purpose the best strains of the respective kind of microorganisms (anaerobic bacteria, aerobic bacteria, and yeast) from earlier trials

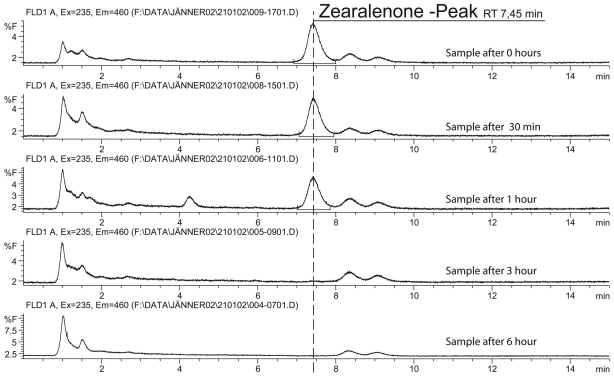

Figure 5. HPLC-spectra of samples which were taken from a zearalenone-degradation study with *T. mycotoxinivoran* (MTV, 115), representing the time course of toxin degradation.

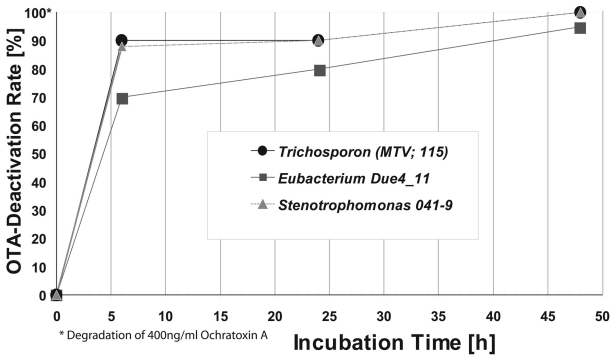

Figure 6. OTA-degradation results of lyophilisates tested in an *in vitro* model with pieces of pig intestines. After 6 h (first sampling time) *T. mycotoxinivorans* (MTV, 115) and *Stenotropomonas sp.* degraded approximately 90% of OTA (400 ng/mL).

were selected. In Fig. 6 results of the comparison study of the study of *T. mycotoxinivorans* (MTV, 115), *Stenotrophomonas* 041-9, and *Eubacterium* Due4_11 are shown. It could be proven that each strain was able to deactivate OTA. This means that even for aerobic microbes enough oxygen was present in this environment to develop their detoxification capabilities. The highest deactivation rates were obtained using yeast and aerobic bacteria. Decrease of OTA-cleavage beginning at 6 h inbucation period could be due to the change of microflora in the intestine (rotting processes). However, the first hour of incubation in such a model represents almost realistic conditions. Therefore this time period is crucial for the evaluation of suitable isolates. Usually resorption of mycotoxins within the intestinal tract takes only a few hours and for this reason highly active strains have to be used to achieve rapid detoxification. Since strain 041-9 showed resistance to many antibiotics, which are used in hospitals (data not shown) *Trichosporon* MTV was selected as the strain with the highest potential to be used as OTA-deactivating feed additive. For a final evaluation feeding trials with this yeast have to be conducted.

De-epoxidation activity of *BBSH* 797 could be verified under conditions of the gut environment within 48 h the active culture developed DON-detoxification activity in all tested gut segments. The best result was obtained in the anterior part of the small intestine; 100% of added deoxynivalenol were transformed resulting in 100% DOM-1. In the respective control samples biotransformation activity could not be detected. From these results it could be assumed that the intestinal environment regarding the gut microflora as well as the physiological conditions including among others pH, redox potential, and oxygen content generally enables biotransformation of DON by *BBSH* 797.

The *in vitro* test model with whole pieces of gut offered one important advantage: The physiological state of the intestine remained less affected in the first hour, wherefore the achieved results might be very close to natural conditions.

3.2 Toxicity testing

These tests were made to show that biotransformation of deoxynivalenol by *Eubacterium BBSH* 797 and ochratoxin A by *T. mycotoxinivorans* (MTV, 115) leads to metabolites that are nontoxic. In the case of DON a LPA assay was used to prove that DOM-1 is less toxic for lymphocytes isolated from chicken than the parent compound DON (see Fig. 7). At a concentration of 0.15 µg/mL proliferation of lymphocytes was lower in comparison with the control (left "black" bar). After adding 0.3 µg/mL to the cells only one-third of them could proliferate whereas at a concentration of 0.63 µg/mL DON the growth of the lymphocytes stopped. In case of the metabolite DOM-1 only a concentration of 116 µg/mL inhibited proliferation of lymphocyte cells completely (see Fig. 7). This test showed that the metabolization of trichothecenes by *Eubacterium BBSH 797* is a detoxification as the metabolite DOM-1 is 500 times less toxic than DON. These data are in accordance with that of Kollarczik *et al.* (1994) [15] who used a MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) test to compare toxicity of DON and its metabolite DOM-1. Detoxification of ochratoxin A by *T. mycotoxinivorans* (MTV) was demonstrated by a MAT. Macrophages were activated with a LPS.

In Fig. 8 the production of NO is shown to be in dependence of the concentration of OTA and its metabolite OTα. It can be seen that in the concentration sector of 0.741 to 2.222 µg/mL OTA, growth of macrophages was depressed. At a concentration level higher than 6.667 µg/mL production of NO, and with that, growth of macrophages, was completely inhibited. In contrast to that the metabolite ochratoxin α does not affect macrophages at a concentration up to 20 µg/mL. Similar results were obtained by Xiao *et al.* (1996) [29]. They compared the toxicity of OTA and

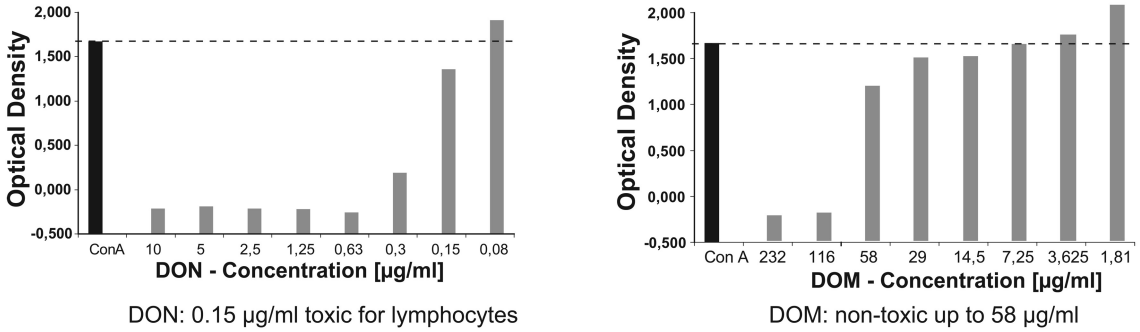

Figure 7. Lymphocytes proliferation test results of DON and DOM-1. DOM-1 is 500 times less toxic than DON.

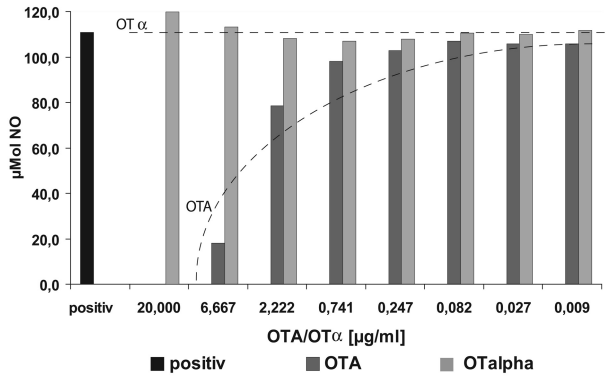

Figure 8. Results of a MAT show that OTα did not inhibit growth of macrophages at a concentration level higher than 6.667 µg/mL, and can be considered as nontoxic.

OTα in prokaryotic (*Bacillus brevis*) and eukaryotic (HeLa cell) systems.

4 Concluding remarks

This study focused on microbial detoxification of certain mycotoxins, like trichothecenes, ochratoxins, and zearalenone, which can hardly be adsorbed to minerals and organic polymers. Therefore these fungal toxins pose a threat for animals receiving bad quality grains.

The presented data show that microbial detoxification by using stabilized microorganisms is a method to counteract these mycotoxins in a very specific and gentle way during the digestion of feed. For deactivation of trichothecenes a bacterial strain (*Eubacterium BBSH 797*) gave promising results; in the case of ochratoxin A the yeast strain *T. mycotoxinivorans* (MTV, 115) has the best properties to be used as detoxifying agent and it could also be shown that this yeast is able to detoxify zearalenone.

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