Monitoring the sterigmatocystin contamination of Hungarian feeds and exploring the biodetoxification opportunities

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Preface

Aflatoxin B1 and sterigmatocystin produced by Aspergillus molds are among the most dangerous genotoxins that endanger food safety. Although sterigmatocystin has been discovered before aflatoxin, there is a very limited range of publications on the production of sterigmatocystin and its biological effects.

In our project, we screened the appearance of sterigmatocystin producing molds in Hungary, the parameters that promote toxin production of the isolates, the biological effect of the mycotoxin, and the feasibility of microbial decontamination.

Scientific results of the project

1. Isolation of the first extreme sterigmatocystin (ST) producer mold in Hungary

1.1. Taxonomical identification

The most important result of the project is, that we were able to isolate and characterize an extreme sterigmatocystin-producing mold strain. During the process *Aspergillus* strains were isolated from Hungarian mills in order to get information on the appearance of sterigmatocystin (ST) producing moulds, whose presence has never been demonstrated in Hungary. Fungal isolates were classified into nine morphotypes, sections *Nigri*, *Nidulantes*, *Versicolores* (two morphotypes), *Circumdati*, *Flavi* (two morphotypes), *Clavati* and *Terrei* by classical mycological assays. ST producing strains could be classified into section *Versicolores*. ST production of the isolates was assessed by liquid and solid phase growth experiments and compared to ST producing reference strains: *Aspergillus pepii* SzMC 22332, *Aspergillus versicolor* SzMC 22333, *Aspergillus griseoaurantiacus* SzMC 22334 and *Aspergillus nidulans* RDIT9.32. One strain, Km26 (former lab code: *Aspergillus versicolor* 2663) of our isolates with the highest ST-production was chosen for the further investigations. Molecular taxonomic identification of the Km26 strain was performed using internal transcribed spacer (ITS), calmodulin and tubulin sequence analyses. Based on these studies, strain Km26 was identified as *Aspergillus creber*.

1.2. Investigating the sterigmatocystin producing ability of A. creber Km26

Four of our isolates marked as Km11, Km14, Km26 and Km31 showed ST production in liquid medium. ST production on solid phase corn grit substrate was measured after three weeks of incubation, and Km26 isolate proved to be the most prominent with a toxin concentration of 277.1 μg g^{-1}, surpassing all reference strains. The *A. creber* Km26 strain produces sterigmatocystin at maximum level under aerobic, solid-phase fermentation conditions. Using high purity corn and rice starch from SIGMA as the only source of carbon, in a solid phase system developed by us, we found that in case of rice starch the toxin level was almost four times higher when compared to corn starch. Scanning electron microscopy studies have shown that rice starch is much smaller in size, with a surface area of the same mass was four times higher than of corn starch - which could be the main factor in the discrepancy.

The toxin-producing ability of Km26 isolate was also tested in a field experiment, where corn was infected. By the end of the experiment, ST level of 19.56 μg kg^{-1} was measured in infected corn. So according to our results ST-producing *A. creber* strain has appeared in Hungary, and the Km26 strain is the first known extreme ST-producing mould in this country. As a result of climate change, aflatoxin B1 producing *Aspergillus flavus* strains have appeared in Hungary in the last decade. As strain Km26 is the only *A. creber* isolate in Hungary so far, there is no sign of mass prevalence, and due to the lower temperature optimum of the species compared to *A. flavus*, its appearance is probably not related to climate change.

2. Microbial ST detoxification

2.1. Sterigmatocystin biodegradation experiments

In the bacterial biodegradation experiments more than 100 different microbe were used. The results showed that the most active aflatoxin degrader rhodococci were unable to degrade ST- It was surprising, that the excellent aflatoxin B1 degrader *Rhodococcus* strains were unable to break down the structurally very similar sterigmatocystin. The best ST-degrader strains belong to *Micrococcaceae*. One of the best ST-degrader with more than 90% degradation was an unknown bacterium species. Strong biodegradation activity was only developed by *Micrococci* and a previously unknown microbe, strain TSL3, with a degradation capability exceeding 90 %.

2.2. Description of new bacterial species with ST-degrading ability

As the most active microbe (TSL3) was an unidentified bacterium we prepared a thorough polyphasic taxonomic identification for it. On the basis of 16S rRNA gene sequence analysis, strain TSL3 is phylogenetically related to the family *Micrococcaceae*. The highest 16S rRNA gene sequence similarity was found with *Micrococcus terreus* V3M1T (96.50 %). Cells of strain TSL3T are aerobic, non-motile and coccoid-shaped. Phenotypic and genotypic characterization clearly showed that strain TSL3T is considerably different from the members of other genera in the family *Micrococcaceae*. According to these results strain TSL3T represents a novel genus and species, for which the name *Micrococcoides hystricis* gen. nov., sp. nov. is proposed.

Also we described an unknown, novel Gram-stain-positive bacterial strain with slight sterigmatocystin degrading activity, designated as K13. On the basis of 16S rRNA gene sequence analysis, the strain showed highest similarity (93.8 %) to *Paenibacillus nanensis* MX2-3T. Cells of strain K13Twere aerobic, motile rods. Based on phenotypic, including chemotaxonomic characteristics and analysis of the 16S rRNA gene sequences, it was concluded that strain K13 represents a novel genus, for which the name Xylanibacillus gen. nov., sp. nov. was proposed.

2.3 Screening for the ST-degrading enzymes of M histricis TSL3 by 2D-electrophoresis approach

Micrococcoides hystricis TSL3 was selected for 2D-proteom analysis, as it was one of the best STC degrader, and we had a draft genome. For 2D-proteom analysis experiment *M. hystricis* TSL3 was cultivated in 250-mL flask which containing 50 mL LB medium (control) and 50 ml LB medium with 1 ppm STC at 30C, 200 rpm for 24 h. After the incubation period cells were collected by centrifugation followed by simultaneous zircon mediated cell lysis and phenol extraction by sonication. For 2D-electrophoresis immobiline dry strips (GE isoelectric points pH 3-11, 18 cm) were used. After isoelectric focusing (IEF), the gel strips were equilibrated and was loaded onto Biorad Tris-Glycine Precast gel, 10%.The proteins were separated at 150 V using BioRad Protean system. From the approx. 1000 different protein spots five overexpressed protein spots were discovered in the toxin containing sample (P1-P5). The spots were cut out from the gel and sent for Maldi-MS protein sequencing to the Max Planck Institute, Germany. According to the protein identification, the overexpressed proteins were: P1- ATP-dependent DNA helicase UvrD2; P2.- Rec A; P3-electron transfer flavoprotein subunit beta; P4-sigle stranded DNA-binding protein; P5-elongation factor TU. According to these results we could not identify potential STC-degrading

enzymes as these proteins seem to participate in DNA-repair mechanism and were induced because of the high genotoxicity of STC.

2.4. Microbial ST-adsorption of lactic acid bacteria (LAB)

Despite the massive quantities of papers dealing with AFB1-binding of lactobacilli, there are no data for microbial binding of the structurally similar mycotoxin sterigmatocystin (ST). In addition, previous works focused on the detection of AFB1 in extracts, while in this case, analytical determination was necessary for the microbial biomass as well. To test binding capacities, a rapid instrumental analytical method using high-performance liquid chromatography was developed and applied for measurement of AFB1 and ST in the biomass of the cultured bacteria and its supernatant, containing the mycotoxin fraction bound by the bacteria and the fraction that remained unbound, respectively. For our AFB1 and ST adsorption studies, 80 strains of the genus *Lactobacillus,* 20 *Enterococcus* strains belonging to *E. casseliflavus, E. faecalis, E. faecium, E. hirae, E. lactis,* and *E. mundtii,* 24 *Pediococcus* strains belonging to species *P. acidilactici, P. lolii, P. pentosaceus,* and *P. stilesii,* one strain of *Lactococcus formosensis* and *L.garviae,* and 3 strains of *Weissella soli* were selected. Broths containing 0.2 μg/mL AFB1and ST were inoculated with the *Lactobacillus* test strains. Before screening the strains for binding capacities, optimisation of the experiment parameters was carried out. Mycotoxin binding was detectable from a germ count of 10^7 cells/mL. By studying the incubation time of the cells with the mycotoxins needed for mycotoxin-binding, co-incubation for 10 min was found sufficient. The presence of mycotoxins did not affect the growth of bacterial strains. Three strains of *L. plantarum* had the best AFB1 adsorption capacities, binding nearly 10% of the mycotoxin present, and in the case of ST, the degree of binding was over 20%.

According to our results, among non-lactobacilli LAB, the genera with the best AFB1 binding abilities were genus *Pediococcus*, with a maximum binding percentage of 7.6% by *P. acidilactici* OR83, followed by genus *Lactococcus*. For AFB1 bio-detoxification purposes, beside lactobacilli, pediococci can also be chosen, but it is important to select a strain with better binding properties than the average value of its genus. Five *Pediococcus* strains have been selected to compare their sterigmatocystin (ST) binding abilities to AFB1 binding, and a 2–3-fold difference was obtained similar to previous findings for lactobacilli. The best strain was *P. acidilactici* OR83 with 18% ST

binding capacity. This is the first report on ST binding capabilities of non-*Lactobacillus* LAB strains.

The toxin adsorption test series provided interesting results: Lactobacilli were able to bind twice as much sterigmatocystin than aflatoxin. This is the first data demonstrating that microbes are able to reduce the amount of ST by adsorption on the cell surface.

3. Monitoring the biological effects of sterigmatocystin

3.1.Genotoxicity of ST measured by SOS-Chromotest and Zebrafish embrio-toxicology

Aflatoxin B1 (AFB1) is the most potent natural carcinogen thus most harmful mycotoxins known. It. has also been shown to be teratogenic and to cause immunosuppression. The most potent AFB1 producers are *Aspergillus flavus* and *A. parasiticus*

Strerigmatocystin (STC) shares its biosynthetic pathway with aflatoxins. *A. nidulans, A. versicolor* and *A. creber* are apparently unable to biotransform STC into O-methylsterigmatocystin, the direct precursor of aflatoxin B1 (AFB1), Consequently, substrates colonized by these fungi can contain high amounts of STC.

While there is abundant data on the biological effects of AFB1, STC is not well characterized mycotoxin with only few publications. As these toxins share the same metabolic pathway their structure is very similar but AFB1 is much more harmful toxin, according to the published data on their biological effects. It has been suggested that sterigmatocystin is about 1/10 as potent mutagenic as aflatoxin B1 measured by Ames test.

In this research the biological effects of STC and AFB1 were examined in two different biomonitoring system.

SOS-Chromotest based on the *E. coli* PQ37 makes use of S9 rat liver homogenate for producing genotoxic epoxide derivatives from AFB1 and STC. Equal concentration of the toxins were measured for genotocicity in intact form and after metabolic activation.

For the second biomonitoring system Zebrafish embryo was chosen because as a model, is widely used in toxicological testing, including embrio-toxicology, since their development is very similar to embryogenesis in higher vertebrates. In the newly developed test S9-bioactivated aflatoxin B1 and sterigmatocystin were microinjected into newly fertilized zebrafish eggs. mortality, sublethal effects and DNA strand breaks were compared on the 5^{th} day of the treatment.

Generally, when comparing the treatments, the activated sterigmatocystin caused the highest mortality and DNA strand breaks in all injected volumes. In the latter case, the sterigmatocystin+S9 treatment cause twice as many of breaks (330,6437 ±(16,07319 DNA_sb μg mg^{-1} protein), than the activated aflatoxinB1 (154,71417 ±(2,91667) DNA_sb μg mg^{-1} protein) in the largest injected volume. e The sublethal symptoms on the embryos were the same for all treatments. The representative development dysfunctions were moderately bent body, not well defined olfactory region, and irregular shaped lower and upper jaw.

The scarce information available suggests that AFB1 is more potent genotoxin:

than STC. Our findings contradict this assumption as in the *E. coli* based SOS-Chromotest the two toxin exert the same genotoxicity. Moreover, according to the newly developed zebrafish monitoring system STC seemed to be more toxic than AFB1. These results raise the demand for use of complex biomonitoring systems for mycotoxin risk assessment.

3.2 Effects of ST on lipid peroxidation and glutathione redox parameters of brojler chickens

The extreme high sterigmatocystin production of the *A. creber* Km26 strain made possible to carry out a broiler chicken feeding experiment using toxin-contaminated grits.

We studied the effect of sterigmatocystin from infected corn (STC), purified sterigmatocystin (PSTC), and aflatoxin B_1 from infected corn (AFB_1) on lipid peroxidation and glutathione redox parameters, including the expression of their encoding genes in a sub-chronic (14 days) trial. A total of 144 three-week old cockerels was divided into four experimental groups (n=36 in each). Control feed was contaminated with STC or PSTC (1590 μg STC/kg or 1570.5 μg STC/kg feed), or with AFB_1 (149.1 μg AFB_1/kg feed). Six birds from each group were sampled at 1^{st}, 2^{nd}, 3^{rd}, 7^{th} and 14^{th} days of mycotoxin exposure. As parameters of lipid peroxidation conjugated dienes (CD) and -trienes (CT) were measured in liver, while malondialdehyde (MDA) concentration in blood plasma, red blood cell hemolysate and liver. Reduced glutathione (GSH) concentration and glutathione peroxidase (GPx) activity was determined in the same samples, and expression of *GPX4*, *GSS* and *GSR* genes was measured by RT-PCR in liver. STC, PSTC or AFB1 caused slight but not significant increase in CD and CT levels, but in case of MDA no increase was found in liver. Glutathione redox system was activated in liver by AFB_1, but less markedly by STC/PSTC. PSTC and AFB_1 resulted higher expression of *GPX4*, while *GSS* expression was down-regulated by AFB_1 on day 1, but up-regulated by STC on day 2 and by both mycotoxins on day 7. However,

on day 14 *GSS* expression was down-regulated by PSTC. Expression of *GSR* was low on day 1 in AFB_1 and PSTC groups, but later AFB_1 up-regulated. Our results show that sterigmatocystin had similar effect to aflatoxin on the detoxification-peroxidation system of broiler chickens.

4. Developing of the genetic basis for ST-research

4.1. De-novo genome project of the ST-producer Aspergillus creber Km26

Based on a de-novo genome project of the strain, we were able to identify the sterigmatocystin synthesis cluster consisting of 26 structure and regulator genes. The *A. creber* Km26 strain produces sterigmatocystin at maximum level under aerobic, solid-phase fermentation conditions (80 ppm on grits and 270 ppm on rice substrates).

We can conclude that sterigmatocystin and extreme high sterigmatocystin producing molds (*A. creber* Km26) have appeared in Hungary. However, this is not related to climate change, as described in case of aflatoxin-producing *A. flavus*. Isolation of *A. creber* was a one-off, special event. The fungus is typically considered as a warehouse mold, but its extreme toxin production on maize and rice substrates indicates that it should be considered as a serious food and feed contaminant in the future.

The results of the project provide a basis for deepening the research on sterigmatocystin. Based on the *A. creber* Km26 de-novo genome project, we were able to design TaqMan tests for the elements of the toxin synthesis cluster so that the physico-chemical and biological parameters of toxin expression could be investigated.

4.2. De-novo genome of the ST-degrader Micrococcoides hystricis TSL3

For de-novo genome project the recently described and efficient ST-degrader Micrococcoides hystricis gen. nov., sp. nov. TSL3 strain was used.For the whole genome sequencing, Nextera Mate Pair Sample Preparation Kit (Illumina) was used. Final libraries were quantified using Qubit (ThermoFisher) and sequenced on an Illumina MiSeq instrument. De novo assembly was performed with CLC Genomics Workbench Tool v10 (Qiagen). Automatic annotation of the genome was performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP). We have assembled the genome of Micrococcoides hystrixii strain TSL3 into 11 contigs with 2,527,433 bps, 2323 putative coding sequences, 48 tRNAs and 3 rRNAs. G+C content

59,63%.According to the whole genome comparisons the most similar genome is from the representative of the Micrococcus genus: Micrococcus luteus SUBG006.The analysis of the annotated genome of TSL3 by PFAM revealed the existence of 23 oxygenases representing mono- and di-oxygenases and P450 enzymes. Micrococcoides hystricis TSL3 is a sterigmatocystin-degrading microbe and the identified oxygenases may take role in the detoxification process. The genome of *M. histricis* (the strain which has been shown to be active in biodetoxification studies) is also available to explore the molecular basis of toxin decomposition.

Summary and perspectives

5.1. The most important result of the project is, that we were able to isolate an extreme sterigmatocystin-producing mold (Km26) from a mill sample originating from the Northern-Hungarian region. According to classic and molecular taxonomic identifications Km26 strain is the first Hungarian representative of the recently described *Aspergillus creber* species. We can conclude that extreme high sterigmatocystin producing molds (*A. creber* Km26) have appeared in Hungary. However, this is not related to climate change, as described in case of aflatoxin-producing *A. flavus*. Isolation of *A. creber* was a one-off, special event. The fungus is typically considered as a warehouse mold, but its extreme toxin production on maize and rice substrates indicates that it should be considered as a serious food and feed contaminant in the future.

5.2. For monitoring the biological effects of ST we used *E. coli* based genotoxicity assay, zebra fish based embrio-toxicity tools and brojler chicken feeding experiments. All the results clearly indicated that ST is equally harmful toxin as aflatoxin B1.

5.3. In the bacterial biodegradation experiments more than 100 different microbe were used. The results showed that the most active aflatoxin degrader rhodococci were unable to degrade ST- which is unusual taking account the strong structural homology of ST- and aflatoxin B1. The best ST-degrader strains belong to Micrococcaceae. One of the best ST-degrader with more than 90% degradation was an unknown bacterium species. We justify that strain TSL3T represents a novel genus and species and described as *Micrococcoides hystricis* gen. nov., sp. nov.

While lactic acid bacteria (LAB) were not able to degrade sterigmatocystin they seem to be very effective toxin binders. Lactobacilli were able to bind twice as much sterigmatocystin than

aflatoxin. This is the first data demonstrating that microbes are able to reduce the amount of sterigmatocystin by adsorption on the cell surface.

5.4. Based on the *A. creber* Km26 de-novo genome project, we were able to design TaqMan tests for the elements of the toxin synthesis cluster so that the physico-chemical and biological parameters of toxin expression could be investigated in the future.

5.5. The genome of *M. histricis* (the strain which has been shown to be active in biodetoxification studies) is also available to explore the molecular basis of toxin decomposition. For this developing special genetic-tools for this taxon also needed (e.g. transposon mutagenesis).

Budapest, 08.03.2021 **Kukolya József**