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INVESTIGATION OF THE FUMONISIN B_1 KINETICS IN PIGS

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1. BACKGROUND OF RESEARCH, OBJECTIVES

Prolonged consumption of mycotoxins in low doses, as an environmental factor, compromises both animal and human health markedly. The healthy organism is able to convert most of the mycotoxins. During this metabolic process the original mycotoxin may be converted to divergent metabolites, resulting from the xenobiotic-transforming activity of the liver and from the conversion by the intestinal microbiota. This metabolic pathway may result more toxic, and biologically more active substances, as compared to the initial molecule.

At present, some metabolites of fumonisin B_1 toxin are known. However, the exact location of transformation of the ingested fumonisin within the gastrointestinal tract is not known. There is a lack of information concerning the absorption and the possibly emerging metabolites of FB_1. Based on studies carried out on mammals is hypothesized that FB_1 mycotoxin is converted to partially or fully hydrolyzed metabolites.

Our research aimed to address the following questions:

- to *prepare fumonisin B_1 toxin* in sufficient quantities, being essential for animal toxicological experiments and
- to investigate:
    1. the toxin absorption from the intestine,
    2. the degree of the biotransformation in the large intestine as a result microbial fermentation,
    3. the effect of low-dose and the long-time exposure on the toxin distribution in the organism and the detection in some organs,
    4. the mode of excretion of the absorbed toxin

  in *in vivo* studies on pigs.

- With the application of an *in vitro* methodology, we aimed to determine the extent of FB_1 transformation during the microbial fermentation in the porcine gastrointestinal tract.

2. MATERIALS AND METHODS

Experiments presented in the Ph.D. thesis can be sub-orded into three main parts from a methodological point of view. However, all of them had a common goal, namely the investigation of the biotransformation of FB_1 in the organism. Studies carried out are summarized below according to the three divergent aspects.

2.1. Fumonisin production under laboratory conditions

Studies were performed to improve the earlier published fumonisin toxin production methods for the strain *Fusarium verticillioides* MRC 826, on the basis of three methods, laying special emphasis on the method of Fazekas (1998). During this process three factors (Factor 1. Quantity of medium: 50 g; Factor 2. Standardized spore suspension from the lyophilized conidia: 10^6 /ml; Factor 3. Water added weekly to flask: 10 ml) were taken into account. These were set to pre-defined values and the toxin production was tested. Sampling was performed in three repetitions of homogenized triplicate jars from all experimental series. To control the weekly increment in the toxin production 3 incubation jars were harvested each time.
At the end of the incubation period (5 weeks), cultures were weighed, air-dried, ground and stored frozen at –18 ^oC until the quantification of fumonisin (HPLC method with fluorescent detection).

2.2. In vivo experiments

The experiments were carried out in the experimental room of the Department of Animal Nutrition of the University of Kaposvár, Faculty of Animal Science. The oral fumonisin load (2-2.2 mg FB_1/kg BW) was performed on weaned barrows (Hungarian Large White) from the age of 8 weeks, in 10 and 22-day trial periods. The main purpose of these investigations was to characterize the effects of continuous toxin load on the toxin localization within the body and on its elimination. Accordingly, after 5-day adaptation period, a *Fusarium verticillioides* fungal culture was mixed into the diet of experimental animals, and the total quantity of urine and faeces was collected either for 5 (experiment I.) or for 22 (experiment II.) days. At the end of the experiments, piglets were sacrificed after sedation and than after gross pathological examinations several organs were sampled. In the second *in vivo* experiment, special T-cannulas (PVTC) were fitted into weaned piglets, in order to determine the absorption of FB_1 from the diet marked by Cr_2O_3 (0.5%). Analyses were carried out by LC-

MS technique (Institute of Animal Hygiene of the Technical University of Munich). In the first experiment only the intact FB_1 and FB_2, while in the second one, also the metabolites of FB_1 (partially hydrolyzed FB_1 [aminopolyol or $PHFB_1$] and aminopentol [AP_1]) were analyzed. Based on the molecular weights of the fumonisin B_1 compounds (fumonisin B_1 (α): 721 g/mol; aminopentol (β): 405 g/mol; aminopolyols (γ): 563 g/mol) the efficiency of the FB_1 conversion into its metabolites was calculated, as described below:

the fumonisin B_1 – aminopentol conversion:

$$\lambda\alpha \rightarrow \beta = \frac{m\beta / M\beta}{m\alpha / M\alpha} = \frac{m\beta}{m\alpha} \times \frac{M\alpha}{M\beta}$$

the fumonisin B_1 – partially hydrolyzed FB_1 conversion:

$$\lambda\alpha \rightarrow \gamma = \frac{m\gamma / M\gamma}{m\alpha / M\alpha} = \frac{m\gamma}{m\alpha} \times \frac{M\alpha}{M\gamma}$$

where *m* indicates the mass of compounds in 1 g and *M* means their relative molecular weights.

The experiments were carried out according to the regulations of the Hungarian Animal Protection Act. The allowance number for the studies was MÁB-11/2002; KÁ-16/2001.

2.3. In vitro experiment focused on the metabolism of fumonisin B_1

In vitro experiments were carried out to determine the metabolism of FB_1 by the intestinal microbiota. For this purpose, suspensions of caecal contents in McDougall buffer solution were incubated anaerobically (37°C) with pure FB_1 (5 μg/ml) for 72 h. The tubes (4 tubes each time) were sampled after 0, 12, 24, 48 and 72 h. The experiment in the above-described form was performed in two repetitions. Mycotoxin analysis was carried out according to chapter 2.2.

2.4. Statistical analysis

To detect between-group differences, oneway ANOVA was used with the LSD (Least Significant Difference) or with Tukey's „post hoc" test, at the probability level of $P \leq 0.05$. Differences between the measured parameters of control and treated groups were compared by independent samples *t*-test ($P \leq 0.05$). Correlation analyses were performed both in the *in vivo* and *in vitro* experiments. SPSS 10.0 was used for the analysis (SPSS Inc., Chicago IL, USA).

3. RESULTS

3.1. Fumonisin production under laboratory conditions

Applying Factors 1, 2 and 3 together, the toxin production started on the first week and continued to increase linearly after this phase. With the developed method, fungal cultures containing high levels of FB_1 (4454 ±624.0 mg/kg FB_1) were prepared. From the fungal cultures the concentrations of both FB_2 and FB_3 toxins were measured, as a complementary examination. It was established that the *Fusarium verticillioides* strain MRC 826 produces the two above-mentioned analogues in that ratio which occurs also in case of the natural contamination. Consequently, the demonstrated method is appropriate for fumonisin toxin production for toxicological experiments on animals.
It has to be emphasized that our aim was not to exceed the results in the literature. In contrast, the work was focused to evolve a simple and cost-effective method, applying the given fungal strain.

3.2. Results of the in vivo experiments

The application of 2 mg FB_1/kg BW did not result clinical symptoms in the experimental animals. The highest toxin concentrations were found in the liver (99.4±11.8 μg/kg) and kidneys (30.6±3.2 μg/kg). Fumonisin B_1 was not detected or found in very low concentrations in muscle and fat samples (muscle: 0.6±0.3 μg/kg; fat: 8.0±4.0 μg/kg), i.e. in the tissue types most widely used for human consumption. On average, 13% of the FB_1 quantity taken up during the 5 days was excreted with the faeces and urine together, about 87% with the faeces and only 13% with the urine. These results related to the unchanged chemical form of FB_1. Therefore – comparing the results to literature data, the low (2-6 %) absorption and rapid (70-90 %) excretion – it was supposed that the major part of the toxin was excreted in partly or totally hydrolyzed form.
Our above-mentioned hypothesis was tested in another *in vivo* experiment. It was established that the accumulative absorption of intact fumonisin B_1 and that of its metabolites formed in the small intestine (until the end of ileum) is 4% in average (maximum: 8.2±1.7%). In the digesta, 95.1% of the total recovered fumonisin B_1 compounds were determined in an intact molecular form, while 1% and 3.9% of this were aminopentol and partially hydrolyzed FB_1, respectively. With respect to the toxin content of organs, much lower FB_1 concentrations (maximum level in the liver) were measured, as compared to those in our earlier experiment. This was probably due to the difference between the daily feed intake and the length of the toxin load in the two trials. In the tissues

investigated, 50% of the recovered FB_1 was chemically unchanged. The efficiency of the FB_1 conversion into aminopentol and partially hydrolyzed FB_1 was 30% and 20%, respectively. In most of the organs, detectable amounts of FB_1 (50%) and its metabolite, aminopentol (50%) were measured even 10 days after the dosage of the non-contaminated diet.

The alteration of the percental ratio of FB_1 to its metabolites in the faeces (**Figure 1**.) after the 5th day was only minor, namely 59% of the total fumonisin B_1 compounds recovered in the faeces was determined as partially (47%) or totally (12%) hydrolyzed metabolites.

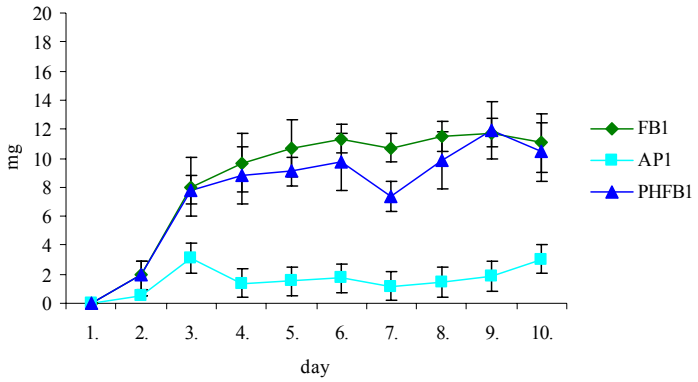

Figure 1 The total amount of fumonisins (mg) recovered in faeces in the period of toxin exposure (n=10)

During the toxin exposure, a relatively close correlation ($r = –0.4$, $P<0.05$) was found between the concentration of FB_1 and $PHFB_1$ in the faeces samples, while there was no statistically significant correlation between the FB_1 and AP_1 concentrations. Three days after the cessation of toxin feeding, $PHFB_1$ was the dominant compound (75%) in the faeces. Detectable amounts of FB_1 and its metabolites were measured in faeces even 10 days after the feeding of the non-contaminated diet.

1.5% of the FB_1 quantity taken up was excreted with the urine, about 65% in original, 16% in totally hydrolyzed and 24% in partially hydrolyzed form, while 23% of the FB_2 consumed during the trial was eliminated by the faeces and 0.6% via the urine, in an unchanged chemical form.

3.3. Results of the in vitro experiment

Along the incubation the hydrolysis of the intact FB_1 form increased; after 48 hours, the conversion of FB_1 into $PHFB_1$ (46%) was near to the percental ratio of

FB_1, while in the 72nd h it reached 49%. Less then 1% of the original FB_1 was converted to aminopentol throughout the 72 h-long incubation (**Figure 2**.).

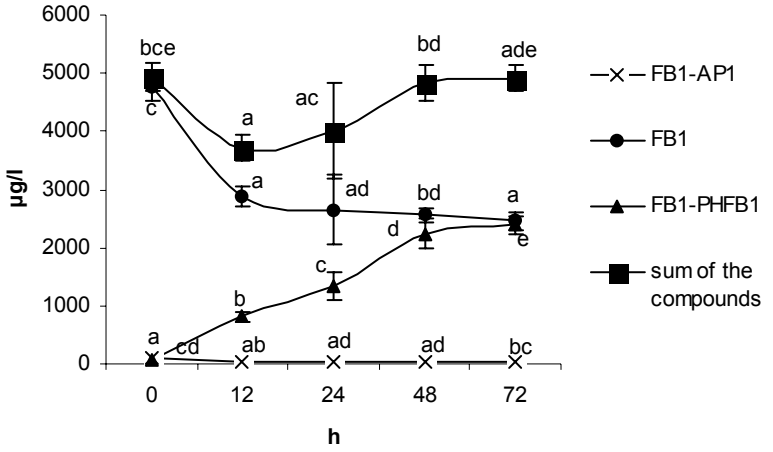

Figure 2 Metabolism of fumonisin B_1 *in vitro* (mean of 8 samples per sampling time)
Values of metabolites are equivalent to that FB_1 amount from which they are originated
Differences in metabolite (AP_1, FB_1, $PHFB_1$ and sum of all compounds, respectively) concentrations at the different sampling times, where different superscripts mean significant difference at $P \leq 0.05$

A relatively close negative correlation ($r = -0.603$; $P \leq 0.05$) was found between the concentration of FB_1 and $PHFB_1$ determined at the different sampling times, while there was no significant correlation between the concentrations of FB_1 and AP_1.

4. CONCLUSION

The fungal strain *Fusarium verticillioides* MRC 826, based on its toxin producing ability has been proven to be applicable for the laboratory-scale **production of fumonisin B_1**. The method developed is proper to grow fungal culture of high toxin concentration for animal experiments. The very high price of pure FB_1 precludes its application in animal feeding trials. A further advantage of the produced fungal culture, as compared to purified FB_1, is that it contains FB_1, FB_2 and FB_3 toxins in similar proportions as in the nature.
The results of the **animal experiments** suggest that high oral doses (ca. 50 mg/kg FB_1) of fumonisins for 10 or 22 days do not induce clinical signs of disease, feed refusal or reduction of the feed intake. Moreover, neither the haematological, nor the clinical chemical blood parameters differed from the physiological reference values. In contrast, pathological alterations were found

both after the 10-day and after the 22-day treatment, primarily in the lung and liver. In the second experiment, even 10 days after the cessation of toxin feeding marked pathological signs were experienced.
In our first *in vivo* experiment on the toxin elimination, by the determination of the intact FB_1 moieties, a strongly negative balance was found in the total toxin amount, when taking the moieties excreted in the urine and faeces into account. This was even true considering the literature data, namely the low (0-6 %) bioavailabilty of fumonisin B_1. This was, at least in part, the basis of our next experiment, where the amounts of the most important and frequent metabolites were also determined.
From the *in vivo* animal experiments complemented with the ***in vitro*** approach, we could draw a conclusion that the intestinal microbiota of pigs is able to transform the intact FB_1 to a similarly toxic substance (**partially hydrolyzed FB_1**) or to a more toxic metabolite (**aminopentol**). As a general conclusion, from the two metabolites, aminopolyol has priority during the metabolic process, *in vivo* and *in vitro*, as well.
The conversion of FB_1 to AP_1 is notable even despite of its little amount, because this new compound means a new risk from the viewpoint of animal- and human health as well, taking into account that aminopentol appears to be tenfold toxic than the FB_1, and that it is hydrophobic molecule (more effective absorption). From the ratio of the marking substance (Cr_2O_3) and FB_1 (and its metabolites) in the digesta, for the calculated accumulative absorption rate (maximally 4%) it is not clear, which metabolites shaped exactly this value.
Since only some 70% of the total FB_1 intake was recovered in the faeces and urine, and the toxin concentration of the divergent organs and tissues was low, the explanation of the negative balance is uncertain. It is hypothesized (1) that a still unknown metabolite emerges, or (2) the toxin may also accumulate in an organ not yet investigated. A further (3) possibility may be that the full elimination of FB_1 and its metabolites requires a much longer period.

Based on the above facts, **further investigations** are reasoned to clarify the role of the small intestinal flora in the biotransformation of FB_1 and to characterize the absorption and toxicity of aminopentol, when administered alone. Moreover, the detailed investigation of the partially hydrolyzed form seems to be also highly important, albeit this is very difficult from technological and analytical aspects. This latter metabolite has not yet been examined in feeding trials, while *in vitro* studies suggest that its toxicity is similar to the original molecule.
Animal experiments on the dose-dependent metabolism and absorption in the field of fumonisin research are clearly justified. The long-lasting (minimally 4 weeks) feeding trials are clearly reasoned, including the determination of all

known metabolites (incl. N-palmitoil-aminopentol); moreover, at least a 4-weeks elimination period would be needed to define the end of toxin elimination. However, the most important task is to seek for new metabolites, and the exact characterization of the *in vivo* metabolism of FB_2 and FB_3 toxins.

5. NEW SCIENTIFIC RESULTS

1. It was established that the accumulative absorption of intact (2-2.2 mg/kg BW) fumonisin B_1 and its metabolites formed in the small intestine (till the end of ileum) is 4% in average in pigs, after oral administration.

2. It was found that 50% of the total accumulated FB_1 is present in an intact form, while 30% is aminopentol and 20% is present as aminopolyol.

3. According to my experiments, during the continuous toxin exposure 60% of the total fumonisin B_1 compounds recovered in the faeces was determined in form of hydrolyzed metabolites, from which the aminopolyol compound appeared in the highest ratio (48% of the sum of all compounds). The ratio of metabolites was much lower in the urine (40%).

4. It was established that most of the porcine tissues, as well as the faeces and urine did contain FB_1 (and its metabolites) 10 days after the cessation of toxin feeding.

5. Incubating pig caecal contents *in vitro* it was found that fumonisin B_1 (5 μg/ml) hydrolysis progressed with the incubation time; after 72 h hours, the molar concentration of partially hydrolyzed toxin was nearly equal with that of intact FB_1, while less that 1% was converted to aminopentol.

6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

6.1. Articles in foreign languages

1. Fodor, J., Bauer, J., Horn, P., Kovács, F., Kovács, M.: Effect of different dietary fumonisin B_1 exposure on the toxin content of porcine tissues. Italian Journal of Animal Science. 2005. 4: 73-78.
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3. Fodor, J., Kametler, L., Kovács, M.: Practical aspects of fumonisin production under laboratory conditions. Mycotoxin Research. 2006. (nyomdában)
4. Fodor, J., Németh, M., Kametler, L., Pósa, R., Kovács, M., Horn, P.: Novel methods of Fusarium toxins' production for toxicological experiments. Acta Agraria Kaposváriensis. 2006. 10. (2):277-285.
5. Kametler, L., Fodor, J., Kovács, M., Horn, P.: Biomarker in the detection of fumonisin toxicosis in pigs. Acta Agraria Kaposváriensis. 2006. 10. (2): 285-291.
6. Fodor, J., Meyer, K., Gottschalk, C., Mamet, R., Kametler, L., Bauer, J., Horn, P., Kovács, F., Kovács, M.: *In vitro* microbial metabolism of fumonisin B_1. Food Additives and Contaminants, 2007. (accepted)

6.2. Articles in Hungarian

1. Kovács, M., Fodor, J., Meyer, K., Mohr, K., Bauer, J., Repa, I., Vetési, F., Horn, P., Kovács, F.: A Fumonizin B_1 kimutatása sertés szöveteiben magas toxintartalmú takarmány etetését követően. Magyar Állatorvosok Lapja. 2004. 126. 146-154.
2. Fodor, J., Meyer, K., Riedlberger, M., Bauer, J., Pósa, R., Horn, P., Kovács, F., Kovács, M..: Fumonizinanalógok Fusarium verticillioides gombatenyészet szájon át való adagolását követő eloszlása a sertés szervezetében és eliminációjuk. Magyar Állatorvosok Lapja. 2006. 128. 334-342.

6.3.Lectures in Hungarian

1. Kovács, M., Meyer, K., Mohr, K., Horn, P., Fodor, J.: A fumonizin B_1 kimutatása sertés szöveteiben magas toxintartalmú takarmány etetését követően. MTA Állatorvos-tudományok Bizottsága, Budapest, 2003.
2. Fodor, J., Kovács, M.: A fumonizin B_1 kimutatása sertés szöveteiben humán egészségügyi kockázat becslése céljából. Ifjúsági Tudományos Fórum, Keszthely. 2004. (CD)
3. Fodor, J., Bauer, J., Horn, P., Kovács, F., Kovács, M.: A fumonizin B_1 forgalmának vizsgálata választott malacokban. MTA Állatorvos-tudományok Bizottsága, Budapest, 2005.
4. Fodor, J., Kovács, M.: A fumonizin B_1 forgalmának vizsgálata választott malacokban. ITF, Keszthely. 2005. (CD)

6.4. Lectures in foreign languages

1. Kovács, M., Fodor, J., Meyer, K., Mohr, K., Bauer, J., Repa, I., Vetési, F., Horn, P., Kovács, F.: Determination of fumonisin B_1 content of porcine tissues after feeding diet of high toxin concentration for the sake of risk assessment. 55th Annual meeting of the European Association for Animal Production. 5-9 September 2004.
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3. Fodor, J., Mamet, R., Kametler, L., Bauer, J., Kovács, M.: In vivo and in vitro metabolism of fumonisin B_1. XXIIth International IUPAC Symposium on Mycotoxins and Phycotoxins, Istanbul, 21-25 May 2007.