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In vitro microbial metabolism of fumonisin B₁

There is a lack of information on the effect of swine caecal microbiota on fumonisin metabolism. In this *in vitro* study, the biotransformation of fumonisin B₁ (FB₁) by the gut microbiota of adult, healthy pigs was examined. For that purpose, suspensions of caecal contents and McDougall buffer solution were incubated anaerobically with pure FB₁ for 0, 12, 24, 48 and 72 h. In the 48ᵗʰ h, the conversion of FB₁ into aminopolyols (46%) was nearly equal to the percental ratio of FB₁, while in the 72ⁿᵈ h it was 49%. *In vitro*, the conversion of fumonisin B₁ to aminopentol was less than 1%. These results show that the caecal microbiota is able to transform fumonisin B₁ to the above metabolites. Further studies on the presence of FB₁ metabolism in the small intestine are clearly justified.

**Keywords:** fumonisin, aminopentol, aminopolyols, biotransformation, metabolites
Introduction

The fumonisin B (FB) analogues, comprising toxicologically important FB₁, FB₂ and FB₃ are the most abundant naturally occurring fumonisins (Marasas, 2000). Fumonisin B₁ (FB₁), the major fumonisin produced by Fusarium verticillioides but seldom also by F. proliferatum (Nelson et al., 1993) both in culture (Alberts et al., 1990) and under natural conditions (Sydenham et al., 1990), is responsible for several toxicological effects in animals. Moreover it has been implicated in the pathogenesis of oesophageal cancer in humans (Voss et al., 2002). Based on research results obtained so far, FB₁ has been evaluated as possibly carcinogenic to humans (class 2B) (IARC, 2002).

From the food safety point of view it is especially important to know the distribution of this mycotoxin in the organism. The toxin concentration in the organs, urine and faeces is influenced by the metabolism of the toxin to a large extent.

The intestinal microbiota plays an important role in the metabolism of mycotoxins. The biotransformation of xenobiotics caused by microorganisms could result in a detoxification as well as a toxication of the parent compound (Rowland, 1981). Only few data have been reported in the literature about the metabolism of FB₁. One of the main FB₁ metabolites, aminopentol (AP₁) appears to be more toxic to rats than FB₁ (Hendrich et al. 1993). N-palmitoyl-AP₁ (derived from AP₁) appears to be considerably more toxic for HT29 cells
than either of the parent fumonisin precursors (Humpf et al., 1998). Thus, if FB$_1$ was
efficiently metabolized to aminopentol even in the small intestine, a mechanism does exist
for metabolic activation (Shier, 2000). However, two cases excepting (Rice and Ross, 1994;
Shephard et al., 1995), in extensive studies on the fate of radiolabeled FB$_1$ administered
orally or by injection, metabolism is generally not detected in the gut or excreta (Shephard
et al., 1992; Norred et al., 1993; Voss et al., 1996; Prelusky et al., 1994, 1996).

Paradoxical data have been reported about the metabolism of FB$_1$ in vitro. However zero
metabolism of FB$_1$ was reported by Prelusky et al. (1996) after 24 h incubation in ruminal
fluid, Smith and Thakur (1996) found that 12.5 and 35% of FB$_1$ was degraded in a buffered
system and 100% ruminal fluid mix, respectively. According to Gurung et al. (1999), there
was minimal (about 10%) degradation of FB$_1$ by ruminal microbes. Whether FB$_1$ was fully
or partially hydrolysed was not known since hydrolysed fumonisins were not determined.
There was no indication that FB$_1$ was metabolized by the human intestinal bacteria, as its
concentration did not decrease in the culture medium during a 72-h incubation (Becker et
al., 1997).

In our earlier study on pigs (Fodor et al., 2006), from the toxin intake of five days, a mean
value of 13% was excreted in urine and faeces as intact FB$_1$. It was supposed that the
major part of the toxin was excreted in a partly or totally hydrolysed form. As swine seems
to be particularly sensitive to the effects of mycotoxins (Bauer et al., 1987), the purpose of
our study was to investigate the involvement of the caecal microbiota in the transformation
of FB$_1$. 
Materials and methods

Materials and chemicals

Caecal content samples were taken immediately after slaughter from adult pigs (n=2; Hungarian large white race) and transferred to the laboratory under anaerobic (Anaerocult box and Anaerocult gas-generator, MERCK, Germany) and sterile condition. Stock solution (0.05 mg ml\(^{-1}\)) of the FB\(_1\) was prepared by dissolving pure FB\(_1\) (F 1147; Sigma-Aldrich, Germany) in sterile distilled water.

Preparation of incubation mixture

One gram of the caecal chyme (contained 1.6 ± 0.23 x 10\(^6\) and 5.8 ± 0.4 x 10\(^8\) Escherichia coli and Bacteroides-species, respectively) was suspended in pre-incubated (24h/37C/anaerobic) McDougall buffer solution (9.8 g NaHCO\(_3\); 9.3 g Na\(_2\)HPO\(_4\)·12H\(_2\)O; 0.57 g KCl; 0.47 g NaCl; 0.12 g MgSO\(_4\)·7H\(_2\)O; 0.04 g CaCl\(_2\) ad 1000 ml aqua dest.; pH 8.3) to yield a 10 % (w/v) suspension. Following a pre-incubation period of 4 h at 37\(^\circ\)C under anaerobic conditions, 1 ml of the 50 mg/kg stock solution of FB\(_1\) was added into the content of each tube, so as to provide a concentration of 5000 ng ml\(^{-1}\) FB\(_1\).

Then suspensions were incubated in an anaerobic cabinet at 37 \(^\circ\)C. The tubes (4-4 tubes derived from the different pigs) were taken off after 0, 12, 24, 48 and 72 h and centrifuged (2000 x g for 20 min), and supernatant fluid and sediment were separated (MLW, Model: Janetzki T23, VEB MLW Zentrifugenbau Engelsdorf, Germany).
Two types of controls were used. One of them was prepared by deep-freezing (-20 °C) the suspended caecal samples immediately after adding FB$_1$ to the suspensions. In order to confirm that caecal samples were not contaminated by other mycotoxins, the others were prepared in the same manner but without the addition of FB$_1$. Moreover from the latter the success of bacteria survival was controlled by a bacteriological examination.

**Mycotoxin analyses**

Intact FB$_1$ and the following metabolites were determined: aminopentol (originates from FB$_1$ by hydrolysis of the tricarballylic acid (TCA) side chains at carbon 14 and 15) and aminopolyols (or partially hydrolysed FB$_1$; it has two forms: PHFB$_1$a - TCA group at the C-15 hydroxy group, namely hydrolysis at C-14; PHFB$_1$b - TCA group at the C-14 i.e. hydrolysis at C-15).

Quantification and identification of fumonisins was carried out using LC (PerkinElmer, Series 200; USA)-MS (API 3200 LC/MS/MS System; Applied Biosystems, USA), based on the method of Fodor et al. (2006).

In case of supernatants, 1 ml of samples was shaken with 2 ml of methanol (30 min), and than the prepared samples were left at room temperature for 10 minutes. After centrifugation (1000 g, 3 min, 20 °C) (Heraeus centrifuge, Model: Megafuge 1.0 R; MERCK, Germany), 1.5 ml of supernatant was mixed with hexane (30 min, vortex) and centrifuged again (1000 g, 3 min, 20 °C). The process was repeated twice, and than the
hexane layer was removed by siphoning and remnant hexane was evaporated in vacuum centrifuge (Christ, Model: RVC 2-25, with a cooling trap from Christ, Model: CT 4-50; Osterode am Harz, Germany).

Sediments (1 g) were extracted by a mixture of 0.1 M ethylenediaminetetraacetate (EDTA) and methanol (8ml; 3/1 v/v) for 60 min. The samples were filtrated through a fiber glass filter (Whatman GF A, Dassel, Germany) after centrifugation (2000 g; 3 min). An aliquot of the filtrate was analysed.

Standards

Pure FB$_1$ (Sigma, F 1147) was used as standard for the determination. PHFB$_1$ standard was prepared by Stephen M. Poling (Mycotoxin Research, National Center for Agricultural Utilization Research, United States Department of Agriculture, Peoria, U.S.A.), according to the method of Poling and Plattner (1999). This standard was an equilibrium mixture of the two partially hydrolysed FB$_1$ forms. AP$_1$ standard for the analysis was made basically by the method of Pagliuca et al. (2005) but with some modification, as described below.

400 µg fumonisin B$_1$ (Sigma, F-1147) were dissolved in 2 ml methanol in a capped vial and than 2 ml of a 1 M KOH-solution was added, and the vial was closed. It was heated in a water bath for 1 h, at 70° C. After cooling down to room temperature the pH was set to 4.5 with a 0.1 M HCl-solution. The solution was extracted with 8 ml of ethyl acetate (two times) by vortexing and short centrifugation. The two ethyl acetate phases were united, and evaporated and than the residues were dissolved in 1 ml of acetonitrile/water (1:1). Because it was not known whether the aminopentol was extracted completely by the ethylacetate,
thus a SPE step of the MeOH/KOH phase followed (lower phase) to remove the KCl-salt which could have disturbed the ionization in the MS. The final elution of the SPE-columns was collected, evaporated, and resolved in 1 ml acetonitrile/water (1:1).

The two 1 ml of extracts from the above mentioned process was measured by MS. Since no FB$_1$ could be detected, we assumed that it was totally converted to aminopentol.

Every standard was diluted with the solution of acetonitrile/water (1:1).

Based on the molecular weight of the fumonisin B$_1$ compounds (fumonisin B$_1$: 721 g/mol; aminopentol: 379 g/mol; aminopolyols: 550 g/mol) the efficiency of the FB$_1$ conversion into the metabolites was calculated, as described below:

\[
\frac{\text{aminopentol (nmol/g)}}{\text{fumonisin B$_1$ (nmol/g)}} = \frac{\text{aminopentol (ng/g)} \times 721 \text{ g/mol}}{379 \text{ g/mol} \times \text{fumonisin B$_1$ (ng/g)}}
\]

\[
\frac{\text{aminopolyol (nmol/g)}}{\text{fumonisin B$_1$ (nmol/g)}} = \frac{\text{aminopolyol (ng/g)} \times 721 \text{ g/mol}}{550 \text{ g/mol} \times \text{fumonisin B$_1$ (ng/g)}}
\]

Statistical analyses

The entire measurement dataset was analysed statistically. Correlation analysis (P ≤ 0.05) was performed to determine the correlation between the concentration of FB$_1$ and PHFB$_1$ or AP$_1$ in samples. Data processing and the mathematical-statistical calculations were performed using the Correlate and Descriptive Statistics modules of the SPSS 10.0 programme package, the SAS System (Local, WIN_PRO) programme package, and the spreadsheet and figure editor programmes of EXCEL 7.0. Statistical evaluation of the results was carried out by analysis of variance (ANOVA) and least significant difference
(LSD) “post hoc” tests. All statements of significance were based on the 0.05 level of probability.

Results and discussion

Figure 1 shows that the concentration of intact FB$_1$ in suspensions sampled after 12 h incubation significantly decreased and in parallel with this decrease, the PHFB$_1$ concentration increased. There was no significant change in the aminopentol concentration during the entire period; it was detected in permanently low concentrations.

The result of an *in vitro* experiment of Gurung et al. (1999) was contradictory to ours, namely the total FB$_1$ concentration increased with the duration of incubation. This was, however, difficult to explain, since the metabolites were not determined. Data from the samples of the different pigs were handled in common. Figure 1 indicates relatively low SD values at every sampling event.

[Insert Figure 1 about here]

Regarding the amount of the total recovered toxins in the supernatant collected at the $12^{th}$ and $24^{th}$ h, a significant decrease was experienced, as compared to the original concentration. Analysis of the sediment part sampled at these timepoints clarified that the unaccounted portion of the FB$_1$ dose appeared in the sediment ($1100 \pm 86$ and $795 \pm 102$ ng
ml\(^{-1}\) at the 12\(^{th}\) and 24\(^{th}\) h, respectively). At the 48\(^{th}\) and 72\(^{nd}\) h the total quantity of the different fumonisin compounds was negligible (<60 ng ml\(^{-1}\)) in the sediment part.

In the 48\(^{th}\) h, the conversion of FB\(_1\) into PHFB\(_1\) (46%) was near to the percental ratio of FB\(_1\), while in the 72\(^{nd}\) h it was 49%. There was no change in the degree of the conversion of FB\(_1\) into aminopentol (<1%) throughout the 72 h-long incubation (Figure 1). The percental ratio of the metabolites in the sediment (in the 12\(^{th}\) and 24\(^{th}\) h) was equal to that in the supernatant.

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 concentration of FB\(_1\) and AP\(_1\).

The low and persisting levels of aminopentol and the increasing values of PHFB\(_1\) suggest that the most probable metabolic process is removal of one propane -1,2,3-tricarboxylic acid side chain by esterase action in the mammalian gut. Additionally, the conversion of FB\(_1\) to AP\(_1\) is not a priority during the metabolism. Although, this conversion (FB\(_1\) to AP\(_1\)) can occur to a lesser extent, as well. The results of our study correlate with the data found after experimental intoxication of animals with FB\(_1\), according to which (Shephard et al., 1995) the partially hydrolysed form (approximately 1/3 of total fumonisins) was the main product in the faeces, with very low amounts of the fully hydrolysed (aminopentol) form recovered. As both the partially and the completely hydrolysed FB\(_1\) could be detected in the
faeces but not in the bile, it was supposed that FB$_1$ is metabolised in the large intestine (Shephard et al., 1995).

If we suppose that the metabolism of FB$_1$ occurs only in the large intestine, it may be assumed that the metabolite (AP$_1$) which is more toxic than the intact FB$_1$ has low bioavailability. There is a lack of information on the toxicity of partially hydrolysed FB$_1$. Caloni et al. (2002) investigated intestinal absorption and toxicity of intact fumonisin B$_1$ and its partially and totally hydrolysed metabolites, using the human intestinal cell line Caco-2. Caco-2 cells were treated for 48 h with several toxin concentrations. At the end of exposure period, no significant variation on cell viability has been observed with all chemicals tested, suggesting a poor toxicity of these mycotoxins for intestinal cells. In any case, FB$_1$ appears the most active in this respect. For which concerns the cellular absorption, FB$_1$, PHFB$_1$a and b are never detected into Caco-2 cells. On the contrary, a dose-dependent absorption of aminopentol has been observed. Thus AP$_1$, losing the tricarballylic acid chains, is more bioavailable than the intact FB$_1$ on intestinal cell, supporting the hypothesis that in risk evaluation of fumonisins exposure its metabolites are also relevant (Caloni et al., 2002).

Although biliary excretion of the $[^{14}\text{C}]-$FB$_1$, $[^{14}\text{C}]-$hydrolysed FB$_1$ (AP$_1$), and $[^{14}\text{C}]-$FB$_1$-fructose was similar after oral administration of those, increased urinary excretion of the $[^{14}\text{C}]-$hydrolysed FB$_1$ as compared to $[^{14}\text{C}]-$FB$_1$ and $[^{14}\text{C}]-$FB$_1$-fructose indicated a greater absorption of the hydrolysed form (Dantzer et al., 1999).
However FB$_1$ has been transformed by the caecal microbiota to aminopentol and partially hydrolysed forms, further studies are needed to clarify the presence of FB$_1$ metabolism in the small intestine.

Conclusion

Based on our findings it can be concluded that the *in vitro* system presented here seems to be well suited to the study the role of swine gut microorganisms in the transformation of fumonisin B$_1$. This observation has not been reported before.

The decrease experienced in the total FB$_1$ concentration at 12$^{th}$ h of the incubation was found because of the unaccounted proportion of the dose appeared in the sediment. Presumably, this decrease in the supernatant was due to the binding of toxin to the cell wall of bacteria. Similar phenomenon was established in case of other mycotoxins, namely cell wall polysaccharide and peptidoglycan were the two main elements responsible for the adhesion of aflatoxin and zearalenone to *Lactobacillus* strains (El-Nezami et al., 1998, 2002).

The concentration of PHFB$_1$ increased continuously, while there was no change in the aminopentol concentration, which was measured in persistently low concentrations throughout the 72 h-long incubation. Summarized, the conversion of FB$_1$ to the measured metabolites was approximately 50%. As a general principle, the most probable metabolic process is removal of one propane –1,2,3-tricarboxylic acid side chain but the conversion of
FB\textsubscript{1} to AP\textsubscript{1} in a little amount may be also possible \textit{in vivo}. The conversion of FB\textsubscript{1} to AP\textsubscript{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\textsubscript{1}, and that it is a hydrophobic molecule (with a more effective absorption) (Humpf et al., 1998). Based on the above facts, further investigations are reasoned to clarify the role of the small intestinal microbiota in the biotransformation of FB\textsubscript{1} and to characterize the absorption of aminopentol, when administered alone. Moreover, the detailed investigation of the partially hydrolised 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 \textit{in vitro} studies suggests that its toxicity is similar to the original molecule (Caloni et al., 2002).

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Figure 1. Concentration of the measured compounds during the 72 h-long incubation period

Values of metabolites are equivalent with the FB₁ amount from which they are hydrolysed.

Different superscripts mean significant difference (P ≤ 0.05) in metabolite (AP₁, FB₁, PHFB₁ and sum of all compounds, respectively) concentrations at the different sampling times.