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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

1 than either of the parent fumonisin precursors (Humpf et al., 1998). Thus, if FB₁ was
2 efficiently metabolized to aminopentol even in the small intestine, a mechanism does exist
3 for metabolic activation (Shier, 2000). However, two cases excepting (Rice and Ross, 1994;
4 Shephard et al., 1995), in extensive studies on the fate of radiolabeled FB₁ administered
5 orally or by injection, metabolism is generally not detected in the gut or excreta (Shephard
6 et al., 1992; Norred et al., 1993; Voss et al., 1996; Prelusky et al., 1994, 1996).

7
8 Paradoxical data have been reported about the metabolism of FB₁ *in vitro*. However zero
9 metabolism of FB₁ was reported by Prelusky et al. (1996) after 24 h incubation in ruminal
10 fluid, Smith and Thakur (1996) found that 12.5 and 35% of FB₁ was degraded in a buffered
11 system and 100% ruminal fluid mix, respectively. According to Gurung et al. (1999), there
12 was minimal (about 10%) degradation of FB₁ by ruminal microbes. Whether FB₁ was fully
13 or partially hydrolysed was not known since hydrolysed fumonisins were not determined.
14 There was no indication that FB₁ was metabolized by the human intestinal bacteria, as its
15 concentration did not decrease in the culture medium during a 72-h incubation (Becker et
16 al., 1997).

17
18 In our earlier study on pigs (Fodor et al., 2006), from the toxin intake of five days, a mean
19 value of 13% was excreted in urine and faeces as intact FB₁. It was supposed that the
20 major part of the toxin was excreted in a partly or totally hydrolysed form. As swine seems
21 to be particularly sensitive to the effects of mycotoxins (Bauer et al., 1987), the purpose of
22 our study was to investigate the involvement of the caecal microbiota in the transformation
23 of FB₁.

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67 **2 Materials and methods**
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1011 4 *Materials and chemicals*
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13
14 5 Caecal content samples were taken immediately after slaughter from adult pigs (n=2;
15
16 6 Hungarian large white race) and transferred to the laboratory under anaerobic (Anaerocult
17
18 7 box and Anaerocult gas-generator, MERCK, Germany) and sterile condition. Stock
19
20 8 solution (0.05 mg ml⁻¹) of the FB₁ was prepared by dissolving pure FB₁ (F 1147; Sigma-
21
22 9 Aldrich, Germany) in sterile distilled water.
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2728 11 *Preparation of incubation mixture*
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33 12
34 13 One gram of the caecal chyme (contained $1.6 \pm 0.23 \times 10^6$ and $5.8 \pm 0.4 \times 10^8$ *Escherichia*
35
36 14 *coli* and *Bacteroides*-species, respectively) was suspended in pre-incubated
37
38 15 (24h/37C/anaerobic) McDougall buffer solution (9.8 g NaHCO₃; 9.3 g Na₂HPO₄·12H₂O;
39
40 16 0.57 g KCl; 0.47 g NaCl; 0.12 g MgSO₄·7H₂O; 0.04 g CaCl₂ ad 1000 ml aqua dest.; pH
41
42 17 8.3) to yield a 10 % (w/v) suspension. Following a pre-incubation period of 4 h at 37°C
43
44 18 under anaerobic conditions, 1 ml of the 50 mg/kg stock solution of FB₁ was added into the
45
46 19 content of each tube, so as to provide a concentration of 5000 ng ml⁻¹ FB₁.
47
48
49 20 Then suspensions were incubated in an anaerobic cabinet at 37 °C. The tubes (4-4 tubes
50
51 21 derived from the different pigs) were taken off after 0, 12, 24, 48 and 72 h and centrifuged
52
53 22 (2000 x g for 20 min), and supernatant fluid and sediment were separated (MLW, Model:
54
55 23 Janetzki T23, VEB MLW Zentrifugenbau Engelsdorf, Germany).
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1 Two types of controls were used. One of them was prepared by deep-freezing (-20 °C) the
2 suspended caecal samples immediately after adding FB₁ to the suspensions. In order to
3 confirm that caecal samples were not contaminated by other mycotoxins, the others were
4 prepared in the same manner but without the addition of FB₁. Moreover from the latter the
5 success of bacteria survival was controlled by a bacteriological examination.

6 7 *Mycotoxin analyses*

8
9 Intact FB₁ and the following metabolites were determined: aminopentol (originates from
10 FB₁ by hydrolysis of the tricarballic acid (TCA) side chains at carbon 14 and 15) and
11 aminopolyols (or partially hydrolysed FB₁; it has two forms: PHFB_{1a} - TCA group at the
12 C-15 hydroxy group, namely hydrolysis at C-14; PHFB_{1b} - TCA group at the C-14 i.e.
13 hydrolysis at C-15).

14
15 Quantification and identification of fumonisins was carried out using LC (PerkinElmer,
16 Series 200; USA)-MS (API 3200 LC/MS/MS System; Applied Biosystems, USA), based
17 on the method of Fodor et al. (2006).

18
19 In case of supernatants, 1 ml of samples was shaken with 2 ml of methanol (30 min), and
20 than the prepared samples were left at room temperature for 10 minutes. After
21 centrifugation (1000 g, 3 min, 20 °C) (Heraeus centrifuge, Model: Megafuge 1.0 R;
22 MERCK, Germany) , 1.5 ml of supernatant was mixed with hexane (30 min, vortex) and
23 centrifuged again (1000 g, 3 min, 20 °C). The process was repeated twice, and than the

1 hexane layer was removed by siphoning and remnant hexane was evaporated in vacuum
2 centrifuge (Christ, Model: RVC 2-25, with a cooling trap from Christ, Model: CT 4-50;
3 Osterode am Harz, Germany).

4
5 Sediments (1 g) were extracted by a mixture of 0.1 M ethylenediaminetetraacetate (EDTA)
6 and methanol (8ml; 3/1 v/v) for 60 min. The samples were filtrated through a fiber glass
7 filter (Whatman GF A, Dassel, Germany) after centrifugation (2000 g; 3 min). An aliquot
8 of the filtrate was analysed.

9 10 *Standards*

11 Pure FB₁ (Sigma, F 1147) was used as standard for the determination. PHFB₁ standard was
12 prepared by Stephen M. Poling (Mycotoxin Research, National Center for Agricultural
13 Utilization Research, United States Department of Agriculture, Peoria, U.S.A.), according
14 to the method of Poling and Plattner (1999). This standard was an equilibrium mixture of
15 the two partially hydrolysed FB₁ forms. AP₁ standard for the analysis was made basically
16 by the method of Pagliuca et al. (2005) but with some modification, as described below.

17 400 µg fumonisin B₁ (Sigma, F-1147) were dissolved in 2 ml methanol in a capped vial and
18 than 2 ml of a 1 M KOH-solution was added, and the vial was closed. It was heated in a
19 water bath for 1 h, at 70° C. After cooling down to room temperature the pH was set to 4,5
20 with a 0.1 M HCl-solution. The solution was extracted with 8 ml of ethyl acetate (two
21 times) by vortexing and short centrifugation. The two ethyl acetate phases were united, and
22 evaporated and than the residues were dissolved in 1 ml of acetonitrile/water (1:1). Because
23 it was not known whether the aminopentol was extracted completely by the ethylacetate,

1 thus a SPE step of the MeOH/KOH phase followed (lower phase) to remove the KCl-salt
2 which could have disturbed the ionization in the MS. The final elution of the SPE-columns
3 was collected, evaporated, and resolved in 1 ml acetonitrile/water (1:1).

4 The two 1 ml of extracts from the above mentioned process was measured by MS. Since no
5 FB₁ could be detected, we assumed that it was totally converted to aminopentol.

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

7
8 Based on the molecular weight of the fumonisin B₁ compounds (fumonisin B₁: 721 g/mol;
9 aminopentol: 379 g/mol; aminopolyols: 550 g/mol) the efficiency of the FB₁ conversion
10 into the metabolites was calculated, as described below:

11 •
$$\frac{\text{aminopentol (nmol/g)}}{\text{fumonisin B1 (nmol/g)}} = \frac{\text{aminopentol (ng/g)} \times 721 \text{ g/mol}}{379 \text{ g/mol} \times \text{fumonisin B1 (ng/g)}}$$

12 •
$$\frac{\text{aminopolyol (nmol/g)}}{\text{fumonisin B1 (nmol/g)}} = \frac{\text{aminopolyol (ng/g)} \times 721 \text{ g/mol}}{550 \text{ g/mol} \times \text{fumonisin B1 (ng/g)}}$$

14 *Statistical analyses*

15 The entire measurement dataset was analysed statistically. Correlation analysis ($P \leq 0.05$)
16 was performed to determine the correlation between the concentration of FB₁ and PHFB₁ or
17 AP₁ in samples. Data processing and the mathematical-statistical calculations were
18 performed using the Correlate and Descriptive Statistics modules of the SPSS 10.0
19 programme package, the SAS System (Local, WIN_PRO) programme package, and the
20 spreadsheet and figure editor programmes of EXCEL 7.0. Statistical evaluation of the
21 results was carried out by analysis of variance (ANOVA) and least significant difference

1 (LSD) “post hoc” tests. All statements of significance were based on the 0.05 level of
2 probability.

3

4 **Results and discussion**

5

6 Figure 1 shows that the concentration of intact FB₁ in suspensions sampled after 12 h
7 incubation significantly decreased and in parallel with this decrease, the PHFB₁
8 concentration increased. There was no significant change in the aminopentol concentration
9 during the entire period; it was detected in permanently low concentrations.

10

11 The result of an *in vitro* experiment of Gurung et al. (1999) was contradictory to ours,
12 namely the total FB₁ concentration increased with the duration of incubation. This was,
13 however, difficult to explain, since the metabolites were not determined. Data from the
14 samples of the different pigs were handled in common. Figure 1 indicates relatively low SD
15 values at every sampling event.

16

17 [Insert Figure 1 about here]

18

19 Regarding the amount of the total recovered toxins in the supernatant collected at the 12th
20 and 24th h, a significant decrease was experienced, as compared to the original
21 concentration. Analysis of the sediment part sampled at these timepoints clarified that the
22 unaccounted portion of the FB₁ dose appeared in the sediment (1100 ± 86 and 795 ± 102 ng

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1 ml⁻¹ at the 12th and 24th h, respectively). At the 48th and 72nd h the total quantity of the different fumonisin compounds was negligible (< 60 ng ml⁻¹) in the sediment part.

In the 48th h, the conversion of FB₁ into PHFB₁ (46%) was near to the percental ratio of FB₁, while in the 72nd h it was 49%. There was no change in the degree of the conversion of FB₁ into aminopentol (<1%) throughout the 72 h-long incubation (Figure 1). The percental ratio of the metabolites in the sediment (in the 12th and 24th 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 FB₁ and PHFB₁ determined at the different sampling times, while there was no significant correlation between the concentration of FB₁ and AP₁.

The low and persisting levels of aminopentol and the increasing values of PHFB₁ 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 FB₁ to AP₁ is not a priority during the metabolism. Although, this conversion (FB₁ to AP₁) can occur to a lesser extent, as well. The results of our study correlate with the data found after experimental intoxication of animals with FB₁, 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 FB₁ could be detected in the

1 faeces but not in the bile, it was supposed that FB₁ is metabolised in the large intestine
2 (Shephard et al., 1995).

3
4 If we suppose that the metabolism of FB₁ occurs only in the large intestine, it may be
5 assumed that the metabolite (AP₁) which is more toxic than the intact FB₁ has low
6 bioavailability. There is a lack of information on the toxicity of partially hydrolysed FB₁.
7 Caloni et al. (2002) investigated intestinal absorption and toxicity of intact fumonisin B₁
8 and its partially and totally hydrolysed metabolites, using the human intestinal cell line
9 Caco-2. Caco-2 cells were treated for 48 h with several toxin concentrations. At the end of
10 exposure period, no significant variation on cell viability has been observed with all
11 chemicals tested, suggesting a poor toxicity of these mycotoxins for intestinal cells. In any
12 case, FB₁ appears the most active in this respect. For which concerns the cellular
13 absorption, FB₁, PHFB₁a and b are never detected into Caco-2 cells. On the contrary, a
14 dose-dependent absorption of aminopentol has been observed. Thus AP₁, losing the
15 tricarballic acid chains, is more bioavailable than the intact FB₁ on intestinal cell,
16 supporting the hypothesis that in risk evaluation of fumonisins exposure its metabolites are
17 also relevant (Caloni et al., 2002).

18
19 Although biliary excretion of the [¹⁴C]-FB₁, [¹⁴C]-hydrolysed FB₁ (AP₁), and [¹⁴C]-FB₁-
20 fructose was similar after oral administration of those, increased urinary excretion of the
21 [¹⁴C]-hydrolysed FB₁ as compared to [¹⁴C]-FB₁ and [¹⁴C]-FB₁-fructose indicated a greater
22 absorption of the hydrolysed form (Dantzer et al., 1999).

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5 1 However FB₁ has been transformed by the caecal microbiota to aminopentol and partially
6
7 2 hydrolysed forms, further studies are needed to clarify the presence of FB₁ metabolism in
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9 3 the small intestine.
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14 **Conclusion**

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18 7 Based on our findings it can be concluded that the *in vitro* system presented here seems to
19
20 8 be well suited to the study the role of swine gut microorganisms in the transformation of
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22 9 fumonisin B₁. This observation has not been reported before.
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27 11 The decrease experienced in the total FB₁ concentration at 12th h of the incubation was
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29 12 found because of the unaccounted proportion of the dose appeared in the sediment.
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31 13 Presumably, this decrease in the supernatant was due to the binding of toxin to the cell wall
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33 14 of bacteria. Similar phenomenon was established in case of other mycotoxins, namely cell
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35 15 wall polysaccharide and peptidoglycan were the two main elements responsible for the
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37 16 adhesion of aflatoxin and zearalenone to *Lactobacillus* strains (El-Nezami et al., 1998,
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39 17 2002).
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46 19 The concentration of PHFB₁ increased continuously, while there was no change in the
47
48 20 aminopentol concentration, which was measured in persistently low concentrations
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50 21 throughout the 72 h-long incubation. Summarized, the conversion of FB₁ to the measured
51
52 22 metabolites was approximately 50%. As a general principle, the most probable metabolic
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54 23 process is removal of one propane -1,2,3-tricarboxylic acid side chain but the conversion of
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1 FB₁ to AP₁ in a little amount may be also possible *in vivo*. The conversion of FB₁ to AP₁ is
2 notable even despite of its little amount, because this new compound means a new risk
3 from the viewpoint of animal- and human health as well, taking into account that
4 aminopentol appears to be tenfold toxic than the FB₁, and that it is a hydrophobic molecule
5 (with a more effective absorption) (Humpf et al., 1998). Based on the above facts, further
6 investigations are reasoned to clarify the role of the small intestinal microbiota in the
7 biotransformation of FB₁ and to characterize the absorption of aminopentol, when
8 administered alone. Moreover, the detailed investigation of the partially hydrolysed form
9 seems to be also highly important, albeit this is very difficult from technological and
10 analytical aspects. This latter metabolite has not yet been examined in feeding trials, while
11 *in vitro* studies suggests that its toxicity is similar to the original molecule (Caloni et al.,
12 2002).

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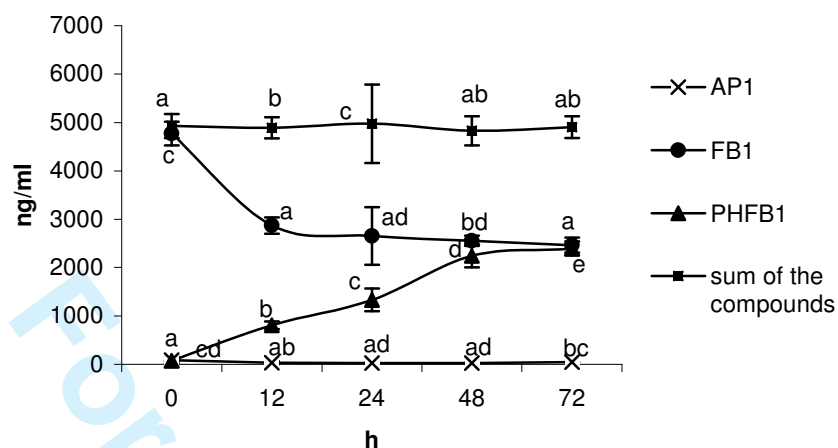
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Figure 1. Concentration of the measured compounds during the 72 h-long incubation period



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

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