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Food and Chemical Toxicology
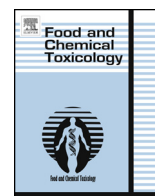
Effects of orally administered fumonisin B_1 (FB_1), partially hydrolysed FB_1, hydrolysed FB_1 and N-(1-deoxy-D-fructos-1-yl) FB_1 on the sphingolipid metabolism in rats

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ARTICLE INFO

ABSTRACT

Fumonisin B_1 (FB_1) is a *Fusarium* mycotoxin frequently occurring in maize-based food and feed. Alkaline processing like nixtamalisation of maize generates partially and fully hydrolysed FB_1 ($pHFB_1$ and HFB_1) and thermal treatment in the presence of reducing sugars leads to formation of N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF). The toxicity of these metabolites, in particular their effect on the sphingolipid metabolism, is either unknown or discussed controversially. We produced high purity FB_1, $pHFB_{1a+b}$, HFB_1 and NDF and fed them to male Sprague Dawley rats for three weeks. Once a week, urine and faeces samples were collected over 24 h and analysed for fumonisin metabolites as well as for the sphinganine (Sa) to sphingosine (So) ratio by validated LC–MS/MS based methods. While the latter was significantly increased in the FB_1 positive control group, the Sa/So ratios of the partially and fully hydrolysed fumonisins were indifferent from the negative control group. Although NDF was partly cleaved during digestion, the liberated amounts of FB_1 did not raise the Sa/So ratio. These results show that the investigated alkaline and thermal processing products of FB_1 were, at the tested concentrations, non-toxic for rats, and suggest that according food processing can reduce fumonisin toxicity for humans.

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1. Introduction

Fumonisins are mycotoxins mainly produced by *Fusarium verticillioides*, *Fusarium proliferatum* (Gelderblom et al., 1988) and *Aspergillus niger* (Frisvad et al., 2007). While several cereal species can be contaminated with fumonisins in principle, the major affected commodity is maize (Placinta et al., 1999). The most commonly occurring form within the family is fumonisin B_1 (FB_1, Fig. 1) and levels of up to several hundred mg/kg can be found in maize (Dutton, 1996). Animal diseases caused by the ingestion of FB_1 include equine leukoencephalomalacia and porcine pulmo-nary oedema. FB_1 is hepatotoxic in all tested species and nephrotoxic in a wide range of animal species, causing apoptosis in the affected tissues (reviewed by Voss et al., 2007). The main mode of action of fumonisins is the inhibition of ceramide synthase and the disruption of the sphingolipid metabolism due to their close similarity to the sphingoid bases sphinganine (Sa) and sphingosine (So) (Merrill et al., 1993a). The elevation of the free Sa/So ratio in urine closely reflected the changes that occurred in kidney of rats exposed to FB_1 (Riley et al., 1994) and was suggested as efficient biomarker of effect. To protect humans and animals numerous countries issued maximum or guidance levels for fumonisins in food and feed. For instance, within the European Union, maximum levels for the sum of fumonisin B_1 and B_2 are 0.2 mg/kg for baby food, 0.8 mg/kg for breakfast cereals or 1.0 mg/kg for maize intended for direct human consumption (European Commission, 2006a). Guidance levels for animals reflect the different toxicities towards different species, with pigs and horses among the most sensitive ones with 5 mg/kg (European Commission, 2006b).

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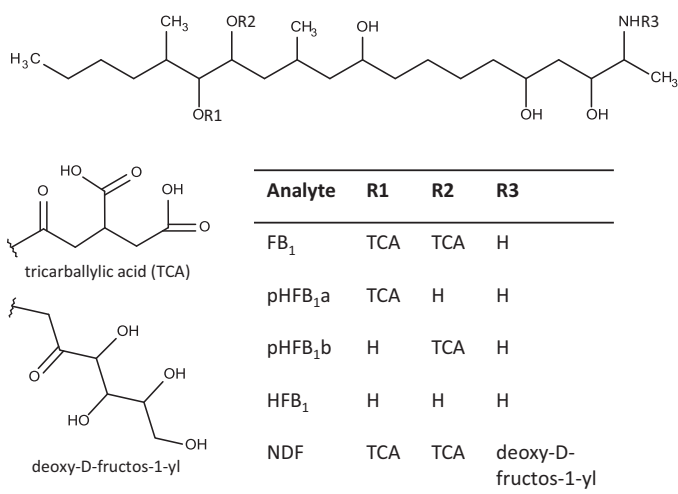
Fig. 1. Structures of fumonisin B_1 (FB_1), partially hydrolysed fumonisin B_1 (pHFB_1a, pHFB_1b), hydrolysed fumonisin B_1 (HFB_1) and N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF).

With a production of 873 million tonnes alone in 2012, maize is the most widely cultivated cereal in the world (FAO, 2014). Maize-based processed food for human nutrition includes cornbread, corn flakes, enchiladas, polenta, popcorn, porridge, quesadillas, steamed corn cobs, tacos and tortillas. Food processing, in particular thermal or alkaline treatment, can lead to derivatisation or cleavage of fumonisins (reviewed by Humpf and Voss, 2004). For instance, N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF, Fig. 1) has been identified as thermal reaction product of FB_1 with glucose (Poling et al., 2002). NDF can further react to form N-(carboxymethyl) fumonisin B_1 (NCM-FB_1) (Howard et al., 1998). As the primary amino group of fumonisins is important for their toxic effects (Gelderblom et al., 1993), these derivatives are regarded as detoxification products (Howard et al., 2002). Still, these compounds might be considered as masked mycotoxins (reviewed by Berthiller et al., 2013), as they might be cleaved during digestion – liberating the precursor mycotoxin. Also alkaline treatment of maize – referred to as nixtamalisation – is extensively performed in several parts of the world, e.g. in Mexico. FB_1 is gradually hydrolysed in this process, as the tricarballylic acid esters at the C-14 and C-15 position are cleaved (Hendrich et al., 1993). The arising products are termed partially hydrolysed fumonisin B_1 (pHFB_1a or pHFB_1b) as well as hydrolysed fumonisin B_1 (HFB_1) and are depicted in Fig. 1. The toxicity of pHFB_1 is poorly investigated and the sole *in vitro* study used rather resistant cell lines towards fumonisins, resulting in little decrease of cell viability even for FB_1 (Caloni et al., 2002). Toxicity trials in rodents and pig showed greatly reduced toxicity of HFB_1 compared to FB_1 when the mycotoxins were administered in feed (Collins et al., 2006; Grenier et al., 2012; Howard et al., 2002) or intraperitoneally (Voss et al., 2009). Reduced toxicity was manifested by no or only weak disruption of sphingolipid metabolism, no observed hepatic pathology in all species tested and no induction of neural tube defects in mice given HFB_1. While Gelderblom et al. (1993) showed no weight loss or induction of cancer by HFB_1, either, the authors noticed similar or even elevated cytotoxicity of HFB_1 compared to FB_1. In addition, feeding trials with fumonisin-containing, nixtamalised fungal culture material showed, depending on treatment conditions and on the starting concentration of intact fumonisins, complete or incomplete (Hendrich et al., 1993; Voss et al., 1996, 1998, 2013) reduction of toxicity. Partially hydrolysed fumonisins, which were suspected to be toxic and present in unknown concentrations in nixtamalised culture material used in early feeding trials, were speculated to be responsible for

contradictory results (Hartinger and Moll, 2011). Another possible reason for partial toxicity of administered nixtamalised culture material might be the *in vivo* formation of N-acyl-HFB_1 (Seiferlein et al., 2007) and potential N-acyl-pHFB_1. HFB_1 can serve as substrate for ceramide synthase, and the resulting cytotoxic N-acyl-HFB_1 is a potent ceramide synthase inhibitor. If HFB_1 was present at sufficiently high concentration for this acylation reaction to occur to a relevant extent, toxicity might be explained.

The aim of the current study was to evaluate the effects of orally administered FB_1, pHFB_1, HFB_1 and NDF on sphingolipid metabolism in rats. Substances were prepared and added to cookies, which were fed to the rodents for three weeks. Urine and faeces were collected weekly for 24 h and biomarkers of exposure and a biomarker of effect were determined using validated LC–MS/MS methods. The study increases the current understanding about sphingolipid metabolism related toxicity and gastro-intestinal metabolism of fumonisin derivatives generated in alkaline or thermal food processing.

2. Materials and methods

2.1. Reagents

Acetonitrile (ACN), methanol (MeOH), glacial acetic acid (HAc) (all LC grade) and aqueous ammonia solution (25%, p.a.) were purchased from VWR International GmbH (Vienna, Austria). Petrol ether (40–60 °C, for chromatography) was purchased from Lactan Roth (Graz, Austria). Water was purified with a Purelab Ultra system (ELGA LabWater, Celle, Germany) after reverse osmosis. Ethyl acetate (EtAc, p.a.) was provided by Wagner & Munz GmbH (Vienna, Austria). All chemicals used for culture media, buffer solutions, as well as silica gel, Amberlite XAD-2 and 10% neutral buffered formalin were purchased from Sigma-Aldrich (Schnelldorf, Germany). Staining solutions for histopathological examinations (haematoxylin and eosin) were obtained from Morphisto (Frankfurt am Main, Germany). Liquid standards of FB_1, [^{13}C]-labelled FB_1, as well as fumonisins B_2 (FB_2) and B_3 (FB_3) were provided by Romer Labs GmbH (Tulln, Austria). Sa and So for calibration were purchased from Avanti Polar Lipids (Alabaster, AL, USA). HFB_1, pHFB_1a, pHFB_1b, NDF as well as a mixture of [^{13}C]-pHFB_1a and [^{13}C]-pHFB_1b were prepared and isolated on a preparative HPLC system according to Schwartz-Zimmermann et al. (in preparation) and Cirlini et al. (in press). [^{13}C]-labelled internal standards of HFB_1 and NDF were prepared from [^{13}C]-FB_1 in the same way at small scale. Single stock solutions of Sa, So, FB_1, pHFB_1a, pHFB_1b, HFB_1 and NDF were prepared in a concentration range between 28 and 100 mg/l. Mixed stocks for preparation of calibration functions were prepared separately for the sphingolipids Sa and So and for all fumonisin analogues at a concentration of 3 mg/l.

2.2. Production and purification of the compounds

F. verticillioides M-3125 (Leslie et al., 1992) was selected as best FB_1 producing strain of our internal strain-database. An agar plug of a six-day old culture on modified Nirenberg-Agar (SNA) (Nirenberg, 1976) was used to inoculate liquid media. Incubation was performed at 25 °C and 70% humidity on a rotary shaker (200 rpm, GFL, Burgwedel, Germany) for 72 h. One millilitre of this fungal culture was added to each baby jar containing approx. 10 g of a mixture of autoclaved milled maize kernels and polenta. After incubation for 28 days (25 °C, 70% humidity) the maize-cultures contained 12.5 g/kg FB_1, 2.2 g/kg FB_2 and 1.3 g/kg FB_3 on average.

Each jar was extracted with 80 ml MeOH/water/HAc (49.5/49.5/1, v/v/v) using an Ultra-Turrax T25 (IKA-Werke, Staufen, Germany) to destroy the mycelium, followed by 90 min on a rotary shaker at room temperature. The extracts were pooled, evaporated to dryness and the remaining oil was dissolved in MeOH/water (25/75, v/v). Amberlite XAD-2 was pre-treated with water (washing), MeOH (conditioning) and MeOH/water (25/75, v/v, equilibration) for 15 min each before the dissolved oil was added. The batch was stirred for 30 min and filtered. Elution of the fumonisins from the residue was performed with the threefold amount of MeOH for 30 min. The filtered extract was evaporated to dryness and dissolved in EtAc/MeOH/HAc (60/39/1, v/v/v) before drying on silica gel. An approx. 700 × 40 mm glass column was packed with silica gel in EtAc/MeOH/HAc (75/24/1, v/v/v) and the fumonisin containing residue was added on top. The column was flushed with 5 l EtAc/MeOH/HAc (75/24/1, v/v/v) and thereafter the fumonisins were eluted with 5 l EtAc/MeOH/HAc (50/49/1, v/v/v) and 5 l EtAc/MeOH/HAc (25/74/1, v/v/v) and collected in two fractions per eluent. The fractions were evaporated to dryness and a preparative HPLC purification was performed for those fractions containing significant amounts of fumonisins. The isolation of all compounds was performed on a preparative 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Sedex LT-ELSD Model 85LT low temperature evaporative light scattering detector (Sedere, Alfortville, France). The column flow was split 1:60 (the minor part transferring into the ELSD and the main part moving to the fraction collector). Purification of FB_1 was

carried out using a Gemini NX C18-column (150 mm × 21.2 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) and a water–MeOH gradient containing 0.1% HAc. The relevant FB_1 fractions were evaporated to dryness, dissolved in water and further diluted for analysis. One FB_1 fraction with a purity of 97% was used for preparation of the cookies for the FB_1 group. Another FB_1 fraction was used for production of HFB_1. To this end, the FB_1 solution was diluted with water and hydrolysis was performed with 2 M KOH in the final solution. After 60 min the conversion was completed and the formed HFB_1 was extracted by liquid–liquid extraction (twice with ACN and twice with EtAc). All organic layers were combined, evaporated to dryness and dissolved in MeOH/water (30/70, v/v). The obtained HFB_1 solution was purified over a Gemini NX C18-column (150 mm × 21.2 mm, 5 µm) and a water–MeOH gradient containing 0.1% HAc using the preparative HPLC system. The relevant fraction was evaporated to dryness, dissolved in water and used for the preparation of the cookies for the HFB_1 group. $pHFB_1a$ and $pHFB_1b$ were synthesised by enzymatic hydrolysis of FB_1 and purified according to Schwartz-Zimmermann et al. (in preparation). NDF was synthesised and repeatedly purified as described by Cirlini et al. (in press). The collected fractions of each substance were pooled and evaporated to dryness on a rotary evaporator at 40 °C. The residues were re-dissolved in pure water. Stock solutions were prepared, quantified and stored at 4 °C until further usage.

2.3. Production and analysis of experimental diets

The experimental diets of the five treatment groups (negative control without toxin, FB_1, $pHFB_1$, HFB_1 and NDF) were produced in 1.5 kg batches. In general, the dough of each batch contained 1.2 kg rat feed previously checked for absence of fumonisins (sniff R/M-H, Ssniff, Soest, Germany), 0.3 kg milled maize, 1 l water and amino acids in solid form (1.52 g/kg L-lysine-HCl, 0.26 g/kg DL-methionine, 0.76 g/kg L-threonine, 0.40 g/kg L-tryptophan and 0.94 g/kg L-valine). For the treatment groups, equimolar amounts of FB_1, $pHFB_1a+b$, HFB_1 and NDF were spiked into the water. Cookies were prepared, dried at 40 °C for three days and stored at –20 °C until 24 h prior to feeding. The analysis of the dried cookies was performed by twofold extraction of 5 g milled cookie with 20 ml each of ACN/water/HAc (79/20/1, v/v/v) for 90 min and LC–MS/MS analysis.

The stability of FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF during preparation and upon storage of cookies was investigated. To that end, cookies were prepared as described above (separately for $pHFB_1a$ and $pHFB_1b$) and analysed after storage times of one week and one month, respectively, and at different temperatures (room temperature, 4 °C, –20 °C and –80 °C).

2.4. Feeding trial

Male Sprague Dawley rats (Hsd:Sprague Dawley SD, five weeks old, 110.8 ± 4.5 g) were purchased from Harlan Laboratories (Udine, Italy). Animals were housed pairwise in Macrolon type III cages (Ehret Life Science Solutions, Emmendingen, Germany) under constant environmental conditions (ventilated cabinets, Ehret Life Science Solutions; 23 ± 1 °C, 55% relative humidity, 12 h light/dark cycle). Enrichment was provided in form of cellulose tunnels (Bioscape, Emmendingen, Germany). Animals had free access to water for the whole duration of the experiment.

Using a randomised block design, rats were assigned to one of the following five groups (n = 4) according to their weight: negative control, FB_1, $pHFB_1$, HFB_1 and NDF. After an acclimatisation period of one week in which control diet was fed to all animals, treatment diets were provided ad libitum for three weeks. With exception of the negative control, diets contained 13.9 µmol/kg of the respective fumonisin derivative, corresponding to 10 mg/kg FB_1, 7.8 mg/kg $pHFB_1$ (3 mg/kg $pHFB_1a$, 4.8 mg/kg $pHFB_1b$), 5.6 mg/kg HFB_1 and 12.2 mg/kg NDF. On days 0, 7, 14 and 21, animals were weighed and kept individually in metabolic cages (Tecniplast, Hohenpeißenberg, Germany) for 24 h to collect urine and faeces. During sampling periods, feed was withdrawn to exclude contamination of excreta by diet derived fumonisins. Urine and faeces samples were volumetrically measured and weighed, respectively, and stored at –20 °C until further analysis. On day 22, animals were euthanised by CO_2 asphyxiation and kidney samples obtained post mortem were frozen at –80 °C.

The animal experiment was approved by the Institutional Ethics Committee and the national authority according to §26ff of the Austrian Law for Animal Experiments, Tierversuchsgesetz – TVG 2012 (BMWV 66.016/0006-II/3b/2013).

2.5. Sample preparation and LC–MS/MS analysis

The methods used for the determination of FB_1, HFB_1, $pHFB_1a$ and $pHFB_1b$ in urine and faeces samples as well as for the determination of Sa and So in urine samples were recently developed and validated by Schwartz-Zimmermann et al. (in preparation). The method for the determination of FB_1 and analogues in urine and faeces samples was additionally validated for analysis of NDF.

For the determination of fumonisin analogues in urine 400 µl sample was mixed with 1200 µl acetone and 16 µl HAc. The mixture was shaken at room temperature for 10 min, sonicated in a water bath for 5 min and centrifuged (2655 × g, 20 °C, 10 min). The supernatants were evaporated to dryness under pressurised air and the residues were taken up in 300 µl ACN/water (30/70, v/v). Finally, the samples were clarified by centrifugation (2655 × g, 20 °C, 10 min), the supernatants were transferred into glass vials and stored at –20 °C until LC–MS/MS analysis. [^{13}C]-labelled recovery standards of FB_1, HFB_1 and NDF were spiked to each urine sample before

work-up. In addition, a mixture of [^{13}C]-$pHFB_1a+b$ was added to an aliquot of sample extract as internal standard prior to measurement for compensation of matrix effects (caused by co-eluting substances which affect ionisation) upon LC–MS/MS analysis.

The sample preparation method for determination of fumonisin analogues in faeces included threefold extraction of 300 mg of homogenised freeze dried faeces with 10 ml, 10 ml and 5 ml of ACN/water/formic acid (74/25/1, v/v/v) by shaking for 30 min, 20 min and 10 min, respectively. After centrifugation (2655 × g, 20 °C, 10 min), the supernatants were combined, vortexed, diluted 1:3 with extraction solvent and centrifuged again prior to LC–MS/MS analysis.

For analysis of Sa and So in urine samples 5 ml aliquots of urine were centrifuged at 2655 × g and 20 °C for 10 min. Subsequently, the pellet was extracted three times with EtAc. The organic phases were pooled, evaporated to dryness under pressurised air and the residues were taken up in 200 µl MeOH/water (80/20, v/v). The samples of the FB_1 group were diluted 1:5 with reconstitution solvent prior to LC–MS/MS analysis. For determination of Sa and So in kidney samples the tissue was homogenised in cold phosphate buffer (50 mM K_2HPO_4) using an Ultra-Turrax T25. MeOH/ACN (50/50, v/v) was added to 200 µl tissue homogenate for protein precipitation, followed by shaking and centrifugation of the mixture. The pellets were extracted with MeOH/water (80/20, v/v), the supernatants were pooled, evaporated to dryness under pressurised air and the residues were taken up in 200 µl MeOH/water (80/20, v/v). The samples of the FB_1 group were diluted 1:10 with reconstitution solvent prior to LC–MS/MS analysis.

LC–MS/MS analysis was performed in the selected reaction monitoring mode on a 1290 Infinity ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies) coupled to a 4000 QTrap tandem mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo V electrospray ionisation (ESI) source in positive ionisation mode. Chromatographic separation was achieved at 25 °C on a reversed phase Gemini C18 column (150 × 4.6 mm, 5 µm particle size) equipped with a 4 mm × 3 mm C18 security guard cartridge. Additionally, enhanced product ion scan (EPI) measurements with a declustering potential of 89 V and collision energies of 35 eV were used to obtain mass spectra of possible NDF-related metabolites or synthesis by-products (m/z 726: partially hydrolysed NDF; m/z 568: hydrolysed NDF; m/z 780: NCM-FB_1; m/z 622: NCM-partially hydrolysed FB_1; m/z 464: NCM-hydrolysed FB_1).

2.6. Histopathological examinations

Liver and kidney samples obtained on day 22 were used for pathological scoring. Tissue was fixed in 10% neutral buffered formalin. Haematoxylin and eosin solution (H&E) was used to stain paraffin tissue sections (5 µm). Liver and kidney tissue was examined with a light microscope, randomly and without knowledge of the group allocation of the samples. Apoptotic cells were measured in the proximal convoluted tubules in the deep cortex and outer medulla. Pathological scoring was performed as described by Riley and Voss (2006).

2.7. Data evaluation

For MS data analysis Analyst 1.6.2 (AB Sciex) was used. Calibration functions for calculation of fumonisin concentrations in urine samples were established by linear regression of peak area ratios (analyte/internal standard) against analyte concentrations. As recoveries of extraction of $pHFB_1a$ and $pHFB_1b$ from urine were close to 100% and matrix effects were compensated by the internal standards, no further correction of the obtained concentrations was required. Due to addition of recovery standard prior to work-up, calculated concentrations of FB_1, HFB_1 and NDF were automatically corrected by the apparent recovery. Concentrations of fumonisins in faeces sample extracts were determined on the basis of linear neat solvent calibration functions. Correction by the apparent recoveries (86–92%) and the dilution factor yielded the concentrations in freeze-dried faeces. Concentrations of Sa and So were calculated from neat solvent calibration functions under consideration of the apparent recoveries (79 and 93%, respectively).

Limits of detection (LOD) and limits of quantification (LOQ) in urine and faeces were calculated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively, on the basis of matrix matched calibration functions. LODs and LOQs in urine were: FB_1 0.3 and 0.9 ng/ml, HFB_1 0.3 and 0.9 ng/ml, $pHFB_1a$ 0.2 and 0.6 ng/ml, $pHFB_1b$ 0.2 and 0.6 ng/ml, NDF 0.7 and 2.4 ng/ml. Consideration of the dilution factor yielded the following LODs and LOQs in freeze dried faeces: FB_1 310 and 940 ng/g, HFB_1 70 and 240 ng/g, $pHFB_1a$ 100 and 290 ng/g, $pHFB_1b$ 220 and 720 ng/g, NDF 220 and 840 ng/g.

Sample preparation of urine and faeces samples was performed in duplicate and average concentrations were calculated. In the case samples from one group showed concentrations below and above the LOQ, LOQ/2 was used for the former samples to calculate average values. The relative standard deviation of work-up and analysis was <20%. The total excreted amounts of FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were calculated based on the volumes of urine and weights of faeces samples, respectively, which were collected from the individual rats per sampling day.

Statistical evaluation was performed using IBM SPSS Statistics 19.0. Comparison of means (body weight, Sa/So ratio) was carried out by analysis of variance with Bonferroni post-hoc test. Results were considered significant at p < 0.05. Statistics of pathological scoring was done with the Kruskal–Wallis test as nonparametric test

and Dunn's test was used for multiple comparisons. Results were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Purity of the compounds

FB_1, HFB_1, as well as $pHFB_1a$, $pHFB_1b$ and NDF were isolated by preparative HPLC in amounts between 30 and 140 mg. The achieved purity of FB_1 was 97.2% (impurities of 1.3% FB_3, 0.6% $pHFB_1a$ and 0.9% $pHFB_1b$), whereas the preparation of HFB_1 did not contain fumonisin related impurities. $pHFB_1a$ showed a purity of 73.2% ($pHFB_1b$ impurity of 26.8%), $pHFB_1b$ a purity of 93.0% ($pHFB_1a$ impurity of 7.0%). The purified NDF contained 2.5% of FB_1.

3.2. Stability test of used compounds during preparation of cookies

Upon preparation of cookies, the recovery for each compound was 70% or higher. However, partially hydrolysed fumonisins converted into each other during drying of the dough to a ratio of $pHFB_1b/pHFB_1a = 1.6$ which then remained stable during storage. Hence, only one group containing both partially hydrolysed fumonisins was included in the subsequent feeding trial. All other compounds (FB_1, HFB_1 and NDF) were stable during drying and storage.

3.3. Feeding trial and histopathological examinations

FB_1 is known to cause several adverse health effects in domestic and laboratory animals (reviewed by Voss et al., 2007). For the present study, the dose of administered FB_1 (and its analogues) as well as the duration of the trial were chosen on the basis of a pre-trial in which cookies containing 10 and 50 mg/kg of FB_1 were fed to Sprague Dawley rats for 4 weeks. Significant elevation of the Sa/So ratio occurred already after one week of exposure in both treatment groups. Based on animal welfare considerations, on the EFSA guidance values for pig (5 mg/kg) and poultry feed (20 mg/kg) and on limited availability of pure fumonisin analogues, the main trial

was performed with 10 mg/kg FB_1 and equimolar concentrations of fumonisin analogues in rat feed.

The different treatment diets had no statistical influence on the final body weight of the animals (data not shown). Likewise, hardly any histopathological effects (pathological scores <0.70 for all groups) were observed for liver and only mild effects were determined for kidney. In kidney, scores of microscopic effects were similar for the negative control group and the NDF group (both <0.60), slightly increased for the HFB1 (1.25 ± 0.46) and pHFB1 (0.88 ± 0.14) group and significantly elevated for the FB1 group (1.56 ± 0.13).

3.4. Fumonisin derivatives in faeces and urine

In the individual sampling periods, volumes of collected urine varied between 4 ml and 61.5 ml per rat, while amounts of freeze-dried faeces ranged from 0.3 g to 2.9 g. Therein, FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were determined by a validated LC–MS/MS based biomarker method. In faeces samples, concentrations of recovered FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were between 4.1–43 µg/g, 1.1–13 µg/g, 1.1–23 µg/g, 0.9–39 µg/g and 27–88 µg/g, respectively. In urine, toxin concentrations exceeding the respective LOQ were only determined for FB_1 (up to 10 ng/ml) and NDF (up to 14 ng/ml). In Tables 1 and 2, the averages for the different treatment groups and sampling time points are shown. For better comparison, data are expressed in molar amounts.

In excreta of the negative control group, none of the fumonisin analogues included in our analytical method was detected. These results are in line with LC–MS/MS analysis of the used rodent diet prior to the start of the experiment which showed absence of all investigated fumonisin analogues as well as of FB_2 and FB_3 (LODs ≤ 7 µg/kg).

In contrast, considerable amounts of FB_1, $pHFB_1a$ and $pHFB_1b$ as well as traces of HFB_1 were detected in faeces of rats dosed with FB_1. In general, partially and completely hydrolysed fumonisins are the only metabolites described to occur after FB_1 exposure in vivo. It is assumed that formation of these metabolites takes place in the digestive tract, most likely realised by gut microbiota (Fodor et al., 2008; Shephard et al., 1995). The extent of FB_1 conversion varies

Table 1
Total amounts of FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF (mean values ± standard deviation (SD)) recovered in faeces of treated rats during a 24 h sampling period. Animals (n = 4) received either blank feed (negative control) or 13.9 µmol/kg diet of the respective fumonisin analogue *ad libitum*.

Group	Day	$FB_1 \pm SD$ (nmol)	$pHFB_1a \pm SD$ (nmol)	$pHFB_1b \pm SD$ (nmol)	$HFB_1 \pm SD$ (nmol)	$NDF \pm SD$ (nmol)
Negative control	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	n.d.	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	n.d.	n.d.
FB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	82.5 ± 19.3	3.57 ± 0.96	2.03 ± 1.14	Traces	n.d.
	14	71.4 ± 32.4	1.98 ± 1.15	1.53 ± 0.47	Traces	n.d.
	21	66.1 ± 36.0	1.48 ± 2.06	2.34 ± 1.82	Traces	n.d.
$pHFB_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	18.9 ± 10.1	32.3 ± 18.3	2.82 ± 1.78	n.d.
	14	Traces	32.5 ± 7.2	57.3 ± 15.0	4.69 ± 5.84	n.d.
	21	Traces	25.3 ± 7.0	46.9 ± 14.6	2.61 ± 1.65	n.d.
HFB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	84.4 ± 58.3	n.d.
	14	n.d.	n.d.	n.d.	90.4 ± 49.3	n.d.
	21	n.d.	n.d.	n.d.	60.9 ± 19.1	n.d.
NDF	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	33.9 ± 11.7	n.d.	n.d.	n.d.	63.8 ± 20.0
	14	26.9 ± 21.4	n.d.	n.d.	n.d.	67.2 ± 28.1
	21	23.6 ± 16.8	n.d.	n.d.	n.d.	96.4 ± 42.2

Notes: FB_1, fumonisin B_1; $pHFB_1a$ and $pHFB_1b$, partially hydrolysed fumonisin B_1; HFB_1, hydrolysed fumonisin B_1; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1; n.d., not detected (analyte concentration in all samples below the limit of detection); traces, at least one sample above the limit of detection, but below the limit of quantification.

Table 2
Total amounts of FB$_1$, pHFB$_1$a, pHFB$_1$b, HFB$_1$ and NDF (nmol, mean ± standard deviation (SD)) recovered in urine of treated rats during a 24 h sampling period. Animals (n = 4) received either blank feed (negative control) or 13.9 µmol/kg diet of the respective fumonisin analogue *ad libitum*.

Group	Day	FB$_1$ ± SD (nmol)	pHFB$_1$a ± SD (nmol)	pHFB$_1$b ± SD (nmol)	HFB$_1$ ± SD (nmol)	NDF ± SD (nmol)
Negative control	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	n.d.	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	n.d.	n.d.
FB$_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	0.11 ± 0.04	n.d.	n.d.	n.d.	n.d.
	14	0.07 ± 0.05	n.d.	n.d.	n.d.	n.d.
	21	0.11 ± 0.05	n.d.	n.d.	n.d.	n.d.
pHFB$_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	Traces	Traces	n.d.	n.d.
	14	n.d.	Traces	Traces	n.d.	n.d.
	21	n.d.	n.d.	Traces	n.d.	n.d.
HFB$_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	Traces	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	Traces	n.d.
NDF	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	Traces	n.d.	n.d.	n.d.	0.07 ± 0.04
	14	Traces	n.d.	n.d.	n.d.	0.08 ± 0.04
	21	Traces	n.d.	n.d.	n.d.	0.11 ± 0.06

Notes: FB$_1$, fumonisin B$_1$; pHFB$_1$a and pHFB$_1$b, partially hydrolysed fumonisin B$_1$; HFB$_1$, hydrolysed fumonisin B$_1$; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B$_1$; n.d., not detected (analyte concentration in all samples below the limit of detection); traces, at least one sample above the limit of detection, but below the limit of quantification.

in dependence of factors like species, route of administration or individual differences (Fodor et al., 2008; Rice and Ross, 1994; Shephard et al., 1994a). In rats, the metabolisation pattern of FB$_1$ has not yet been completely elucidated. Shephard et al. (1992) reported the vast majority of orally administered FB$_1$ being excreted unmetabolised in faeces. In contrast, Hopmans et al. (1997) proposed that approximately 20% of the toxin dose undergoes metabolic transformation in the gastrointestinal tract. Unfortunately, details on the structure of produced metabolites were not presented in that study. Applying state-of-the-art analytical techniques, we were able to extend the current knowledge on FB$_1$ metabolisation based on hydrolysis in rats. In the present study, FB$_1$, pHFB$_1$a, pHFB$_1$b and HFB$_1$ reflected 93.8 ± 2.4%, 3.1 ± 2.4%, 2.8 ± 1.4% and 0.3 ± 1.4%, respectively, of the total amount of detected FB$_1$ analogues in faeces of rats fed the FB$_1$ diet (average of all sampling time points). Quantifiable concentrations of pHFB$_1$a, pHFB$_1$b and traces of HFB$_1$ could be found in faeces samples of all animals, albeit not at all sampling time points. Although a favoured formation of pHFB$_1$a in comparison to pHFB$_1$b was described in faeces of vervet monkeys (pHFB$_1$a:pHFB$_1$b, 83:17; Shephard et al., 1995), trans-esterification by intramolecular rearrangements was suggested to lead to a final equilibrium of 45:55 (Shephard et al., 1994b). In faeces of our rats, considerable variations of this proportion, reaching from 9:91 to 78:22, were observed between individual animals and different sampling time points. Seemingly, speed of analysis as well as individual differences in the gut microbiome have a strong influence on this ratio. In urine, comparably low levels of FB$_1$ were determined. The lack of urinary pHFB$_1$a, pHFB$_1$b and HFB$_1$ points to a diminished relevance of hydrolysed fumonisins after oral FB$_1$ exposure in rats.

To the best of our knowledge, the metabolism of partially hydrolysed fumonisins has not been investigated *in vivo* so far. In rats dosed with pHFB$_1$a+b, the main faecal metabolites were found to be pHFB$_1$a and pHFB$_1$b. Amounts of pHFB$_1$b clearly exceeded those of pHFB$_1$a, leading to a proportion pHFB$_1$a:pHFB$_1$b of 36:64 in the faeces samples. This ratio very much resembles the proportion of partially hydrolysed fumonisins in the provided diet (pHFB$_1$a:pHFB$_1$b, 38:62). In addition, HFB$_1$ and traces of FB$_1$ were detected in faeces samples of this group. The amount of HFB$_1$ generated from pHFB$_1$a+b was similar to the amount of pHFB$_1$a+b generated from FB$_1$, which

indicates that intact and partially hydrolysed fumonisins are hydrolysed at similar rates. In urine of pHFB$_1$ dosed rats, only traces of pHFB$_1$a und pHFB$_1$b were found, thus indicating a low bioavailability of these analogues or further metabolisation.

In rats receiving the HFB$_1$ contaminated diet, exclusively HFB$_1$ was detected in faeces and urine. Previously, significant differences between HFB$_1$ concentrations in hydrolysed and non-hydrolysed faecal extracts of rats dosed with HFB$_1$ were observed (Hopmans et al., 1997). Authors therefore assumed an extensive metabolic conversion of this derivative. The high levels of faecally excreted HFB$_1$ in our experiment (similar levels compared to FB$_1$ treatment) do not corroborate these findings in rats. The presence of trace amounts of HFB$_1$ in urine of only one animal is notable, since a higher bioavailability of HFB$_1$ in comparison to FB$_1$ was suggested in former studies (Caloni et al., 2002; Dantzer et al., 1999; Hopmans et al., 1997). Metabolisation of HFB$_1$ after absorption from the gastrointestinal tract would explain the lack in urinary detected metabolites in our study. For example, the formation of *N*-acyl-metabolites was demonstrated after *intraperitoneal* HFB$_1$ administration (Seiferlein et al., 2007). If any such products were formed in our study, concentrations would have been too low to affect ceramide synthase activity. Furthermore, conjugation processes with sulphate (as proposed by Hopmans et al., 1997) or glucuronic acid are feasible. Thus, a future task will be the identification of such metabolites using high resolution mass spectrometry.

Masked mycotoxins like NDF pose a risk to human and animal health either by exerting biological effects on their own or by liberating their parent forms during mammalian digestion. Studies investigating the latter aspect came up with ambiguous results. While *in vitro* experiments showed that NDF is rather stable under conditions imitating the human (non-microbial) digestion (Falavigna et al., 2012), *in vivo* hydrolysis was proposed for rats (Hopmans et al., 1997). In the current experiment, NDF reflected the major faecal metabolite of animals exposed to the NDF contaminated diet. In addition, minor amounts of FB$_1$ were detected. The average proportion NDF:FB$_1$ (79.5:20.5) in faeces samples differed markedly from the one in the provided diet (97.5:2.5). We therefore conclude that faecally excreted FB$_1$ derives from partial cleavage of NDF in the gastrointestinal tract rather than from impurities in the feed. It can be

speculated that species specific differences in the gut microbiome play an important role regarding the extent of cleavage of this masked mycotoxin during digestion. Besides NDF and FB_1, no other NDF-related metabolites, in part described in the literature by Howard et al. (1998) and Poling et al. (2002) (partially hydrolysed NDF, hydrolysed NDF, NCM-FB_1, NCM-pHFB_1 and NCM-HFB_1), were detected in faeces samples. In urine of NDF exposed rats, NDF as well as traces of FB_1 were determined. The urinary excretion of intact NDF is contradictory to earlier findings, where the presence of merely metabolised NDF was suggested (Hopmans et al., 1997). Trace levels of urinary FB_1 indicate a very limited absorption of this toxin from the gastrointestinal tract.

In contrast to most of the previous toxicokinetic studies performed in rats (Dantzer et al., 1999; Hopmans et al., 1997; Shephard et al., 1992), we did not administer the toxins by gavage, but mixed them into the diet. To avoid bias in urine and faeces sample analysis due to diet derived fumonisins, feed was withdrawn for duration of the individual sampling periods. In consequence, excreted toxin amounts could not be correlated to amounts of ingested toxin and statements on the bioavailability of administered fumonisin analogues are accompanied by some uncertainties. Still, based on overall recovered toxin amounts in excreta, the proportion of analytes detected in faeces exceeded 99% in all treatment groups and on all sampling time points. Although biliary excretion of absorbed fumonisins after oral administration has to be taken into consideration to some minor extent (Dantzer et al., 1999; Shephard et al., 1994c), our data confirm the generally low bioavailability of fumonisins (reviewed by Voss et al., 2007). Further evidence for differences in the absorption rates of certain analogues (Dantzer et al., 1999; Hopmans et al., 1997) cannot be provided on basis of our results.

3.5. Sa/So ratios in urine and kidney

In animal models, the Sa/So ratio serves as specific biomarker for the evaluation of fumonisin toxicity (summarised by Riley et al., 2011). However, differences in organ sensitivity between species, strains and even sex have to be taken into consideration. For example, FB_1 exposure leads to increased Sa/So ratios in liver and plasma of pigs (Grenier et al., 2012), while in most rat strains (e.g. Sprague Dawley) the nephrotoxic effects of fumonisins are more prominent (Riley and Voss, 2006). Average Sa/So ratios in urine of the different treatment groups and sampling time points are provided in Table 3. Urinary Sa/So ratios of the negative control group did not increase over the whole duration of the trial. On the contrary, a disruption of sphingolipid metabolism in rats exposed to FB_1 was observed as early as day 7. Thereafter, levels of Sa/So stayed relatively constant, with Sa and So reaching absolute concentrations as high as 6100 ng/ml and 880 ng/ml in urine, respectively.

While the negative impact of FB_1 on sphingolipid metabolism has been extensively reviewed, reports on the *in vivo* toxicity of hydrolysis products of FB_1 are either controversial (in case of HFB_1) or non-existing at all (in case of $pHFB_1a$ and $pHFB_1b$). Most of the studies on HFB_1 demonstrated a much lower toxicity in comparison to FB_1 (summarised by Humpf and Voss, 2004). Yet, certain effects of HFB_1 on the Sa/So ratio and on cancer promoting activity were observed in experiments where nixtamalised corn culture material was fed to rats (Hendrich et al., 1993; Voss et al., 1998). As possible reasons, the presence of matrix-bound fumonisins (Seiferlein et al., 2007) or partially hydrolysed fumonisins (Grenier et al., 2012) in administered culture material have been suggested. Burns et al. (2008) could indeed present evidence for interactions between FB_1 and the corn matrix during the nixtamalisation process. However, neither in the mentioned study, nor in a recent experiment conducted by Voss et al. (2013) significant impact of matrix-bound fumonisins on the sphingolipid metabolism in rats could be demonstrated. In contrast, only *in vitro* data are available concerning the toxicity of partially hydrolysed fumonisins (Caloni et al., 2002). Unfortunately, the cell line used in this study was rather insensitive to fumonisin exposure (independent of the tested analogue) and therefore conclusions on toxicity are limited. The two $pHFB_1$ variants might be expected to act as potent ceramide synthase inhibitors, because based on the results by Humpf et al. (1998), one TCA side chain should be enough to block the acyl-CoA binding site of ceramide synthase, as was reported for the *Alternaria alternata* f.sp. *lycopersici* AAL toxin (Abbas et al., 1994; Merrill et al., 1993b). In the present experiment, neither the exposition to HFB_1 nor to $pHFB_1$ induced significantly elevated Sa/So ratios in urine of treated animals. Based on these results, we suppose that HFB_1 and $pHFB_1$ are both significantly less toxic than FB_1. Admittedly, rats of the $pHFB_1$ group received a diet containing a mixture of $pHFB_1a$ and $pHFB_1b$ and hence, conclusions on toxicity of the individual forms are impaired. If one but not the other $pHFB_1$ variant is a ceramide synthase inhibitor, its concentration in the present study was too low to affect the sphingolipid biomarker. Nevertheless, no other impurities were detectable in the feed. Thus, our results do not confirm the assumption of partially hydrolysed fumonisins being the causative agent of negative health effects observed in former studies using nixtamalised corn material.

NDF, one of the major products formed by extrusion cooking of FB_1 in the presence of reducing sugars, is supposed to possess a lower toxicity than FB_1 due to blockage of the primary amino group (Howard et al., 2002). In the past, several studies aiming to evaluate the toxicity of reaction products of FB_1 and fructose/glucose have been performed (Fernández-Surumay et al., 2004, 2005; Howard et al., 2002; Liu et al., 2001; Lu et al., 1997). In general, a reduced toxicity of FB_1-glucose/fructose-adducts in comparison to FB_1 was reported (reviewed by Jackson et al., 2012). However, with exception of Howard et al. (2002), who fed NCM-FB_1 of high purity to rats, the reaction products in the administered diets were either not characterised or contained significant levels of unreacted FB_1. These drawbacks were discussed as reasons for toxicity signs still

Table 3
Urinary sphinganine-to-sphingosine ratios (Sa/So, mean ± standard deviation (SD)) of different treatment groups (n = 4) and sampling time points. Sa/So ratios with the same letter are not significantly different from each other (Bonferroni *post-hoc* test, p < 0.05).

Group	Sa/So d0 (mean ± SD)	Sa/So d7 (mean ± SD)	Sa/So d14 (mean ± SD)	Sa/So d21 (mean ± SD)
Negative control	0.17 ± 0.01	0.26 ± 0.12[b]	0.26 ± 0.06[b]	0.27 ± 0.11[b]
FB_1	0.22 ± 0.07	6.10 ± 3.15[a]	6.00 ± 1.57[a]	6.68 ± 1.78[a]
$pHFB_1$	0.16 ± 0.02	0.19 ± 0.04[b]	0.16 ± 0.02[b]	0.16 ± 0.03[b]
HFB_1	0.17 ± 0.07	0.19 ± 0.02[b]	0.17 ± 0.02[b]	0.18 ± 0.04[b]
NDF	0.18 ± 0.06	0.21 ± 0.03[b]	0.23 ± 0.06[b]	0.30 ± 0.08[b]

Notes: FB_1, fumonisin B_1; $pHFB_1a$ and $pHFB_1b$, partially hydrolysed fumonisin B_1; HFB_1, hydrolysed fumonisin B_1; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1.
Symbols "a" and "b" refer to statistical differences between groups (as indicated in the table description).

observable after administration of FB_1-glucose/fructose-adducts (e.g. enhanced tumour cytotoxicity, development of porcine pulmonary oedema, elevated Sa/So ratios) in some of these studies (Fernández-Surumay et al., 2004, 2005; Lu et al., 1997). In the present study, NDF of high purity, obtained by preparative HPLC purification and determined by LC–MS/MS methods, was used. In rats receiving the respective diet, no increase in urinary Sa/So ratios was detected. NDF itself had no effect on sphingolipid metabolism, and FB_1 was liberated from NDF at too low concentration or too far down the digestive tract to elicit toxicity. We therefore suggest that NDF is less toxic than FB_1 in rats.

Sa and So concentrations were also determined in kidney samples. Average Sa/So ratios in the negative control, FB_1, $pHFB_1$, HFB_1 and NDF group reached levels of 0.12 ± 0.01, 4.06 ± 0.63, 0.14 ± 0.03, 0.12 ± 0.01 and 0.23 ± 0.09, respectively. These results, demonstrating a statistically significant elevation of the Sa/So ratio only in kidney samples of rats exposed to FB_1, are in good agreement to data obtained for urine.

3.6. Conclusions

Partially hydrolysed FB_1, HFB_1 and NDF are major food and feed processing products of FB_1. In addition, as revealed in the present study, $pHFB_1$ and HFB_1 are natural hydrolysis products of FB_1 in rats, most likely formed by intestinal microorganisms. By producing high purity $pHFB_{1a+b}$, HFB_1 and NDF and feeding these substances to rats, we were able to extend the current knowledge on the toxicological relevance of these compounds. Whereas the Sa/So ratio in urine and kidney of the FB_1 group was tremendously elevated even after one week of exposure to 10 mg/kg FB_1 in the diet, neither $pHFB_1$ nor HFB_1 increased the Sa/So ratio. Analysis of fumonisin metabolites in rat faeces and urine by validated LC–MS/MS based methods indicated that NDF is partly cleaved to FB_1 during gastrointestinal passage. Yet, the liberated FB_1 was mainly excreted in faeces and did not raise the Sa/So ratio. Hence, all investigated fumonisin derivatives are of much lower toxicological relevance than FB_1. These insights into gastro-intestinal metabolism and toxicology of fumonisin derivatives are important contributions to food and feed safety.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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