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Targeted sphingolipidomics indicates increased C22-C24:16 ratios of virtually all assayed classes in liver, kidney, and plasma of fumonisin-fed chickens

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ABSTRACT
The biological properties of sphinganine-(d18:0), sphingosine-(d18:1), deoxysphinganine-(m18:0), deoxy-
-
sphingosine-(m18:1), deoxymethylsphinganine-(m17:0), deoxymethylsphingosine-(m17:1), sphingadienine-
(d18:2), and phytosphingosine-(t18:0)-sphingolipids have been reported to vary, but little is known about the
effects of fumonisins, which are mycotoxins that inhibit ceramide synthase, on sphingolipids other than those
containing d18:0 and d18:1. Thirty chickens divided into three groups received a control diet or a diet containing
14.6 mg FB1 + FB2/kg for 14 and 21 days. No effects on health or performance were observed, while the effects
on sphingoid bases, ceramides, sphingomyelins, and glycosylceramides in liver, kidney, and plasma varied. The
t18:0 forms were generally unaffected by fumonisins, while numerous effects were found for m18:0, m18:1, d18:2,
and the corresponding ceramides, and these effects appeared to be similar to those observed for d18:0,
and d18:1-ceramides. Partial least square discriminant analysis showed that d18:1- and d18:0-sphingolipids are
important variables for explaining the partitioning of chickens into different groups according to fumonisins
feeding, while m17:1-, m18:0-, m18:1-, d18:2-, and t18:0-sphingolipids are not. Interestingly, the C22-C24:C16
ratios measured for each class of sphingolipid increased in fumonisin-fed chickens in the three assayed matrices,
whereas the total amounts of the sphingolipid classes varied. The potential use of C22-C24:C16 ratios as bio-
markers requires further study.

1. Introduction
Fumonisins are mycotoxins found in foodstuffs in many countries (Chen et al., 2021; Gao et al., 2023). These compounds are toxic to animals, are known to be carcinogenic in rodents, and are classified in group 2B as possibly carcinogenic to humans (IARC MONograph 82, 2002). The toxic manifestations of fumonisins are multiple, and both the species sensitivities and the target organs vary greatly, but the reason of these variations remains largely unknown (Chen et al., 2021; EFSA et al., 2018; EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2022; Gao et al., 2023). Nevertheless, inhibition of ceramide (Cer) synthesis by blocking ceramide syntheses (CerS) is recognised as a key step in the mechanism of action of fumonisins (Fig. 1) (Riley and Merrill, 2019). This inhibition leads to blockage of de novo synthesis of sphingolipids and the accumulation of sphinganine (Sa). Hydrolysis of membrane sphingolipids can occur secondarily, leading to partial restoration of cellular ceramide levels and an increase in sphingosine (So) concentrations through the salvage pathway (Fig. 1) (Riley and Merrill, 2019). Consequently, the Sa:So ratio is used as a biomarker of fumonisins in humans and many animal species. However, it is accepted that change in Sa:So is not sufficient to explain fumonisin toxicity (Riley and Merrill, 2019).

A key point that could explain fumonisin toxicity is their effects on ceramides, and notably differential effects according to the carbon chain length of the incorporated fatty acid. Very-long-chain ceramides, for which the fatty acid has 22–26 carbons (C22-C26), are mainly synthesized by CerS2 (Cingolani et al., 2016; Ho et al., 2022; Mullen et al., 2012). Long-chain ceramides, for which the fatty acid has 14–16 carbons (C14-C16), are synthesized by CerS5 and CerS6 (Cingolani et al., 2016; Ho et al., 2022; Mullen et al., 2012). Mouse models have revealed that a deletion in CerS2 leads to a reduction in very-long-chain Cer and a 'compensatory' increase in long-chain Cer, which reproduced the signs

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nc-nd/4.0/).
of hepatotoxicity observed in rodents exposed to fumonisins (Pezzner-Jung et al., 2010b, 2010a). Additionally, a decrease in C22-C24-ceramides has been observed in the lung, but not the liver, of pigs fed fumonisins, a species that is highly sensitive to pulmonary oedema due to fumonisin exposure (Loiseau et al., 2015). In contrast, recent studies in chickens and turkeys, which are considered to be relatively resistant to fumonisins, have revealed a decrease in the long-chain ceramides and an increase in the very-long-chain ceramides in the liver and kidneys (Guerre et al., 2022b, 2022c, 2022a; Tardieu et al., 2021). Studies in chickens are therefore of interest because, as they are considered to be relatively resistant to fumonisins, specific changes in their sphingolipidome may help to explain the differences in toxicity between species. Finally, all these studies suggest that the effects of fumonisins on ceramides vary according to the species and tissue, and these variations could be important in explaining toxicity.

Another hypothesis to explain fumonisins toxicity relates to their effects on sphingolipids that are of low abundance in cells but that have high biological activity. This hypothesis was first espoused for deoxy- ceramides (Duan and Merrill, 2015; Jimenez-Rojo et al., 2014; Zitomer et al., 2009). Deoxyceramides contain deoxysphinganine (m18:0), which is synthesized in cells by the incorporation of alanine instead of serine (Fig. 1). Deoxyceramides are now recognized to be toxic compounds and are associated with obesity and inflammatory responses (Hannich et al., 2021; Lauterbach et al., 2021). In the same way, as a result of the absence of the C4-C5 double bond in sphinganine, dihydroceramides and dihydro sphingomyelins have specific roles in cell survival and lipid rafts (Kinoshita et al., 2020; Lachkar et al., 2021). Dihydroceramides, long considered to have weak biological properties, have recently been reported to be associated with steatosis and fibrosis in non-alcoholic fatty liver disease (Babiy et al., 2023; Savira et al., 2021). Deoxymethyl sphinganine (m17:0) is synthesized in cells by the incorporation of glycine instead of serine (Fig. 1) (Ikushiro et al., 2023), and m17:0- and m17:1-ceramides could play a role in neurodegenerative and cardiovascular disorders (Di Pietro et al., 2023; Rossor and Reilly, 2022). Sphingadename (d18:2), potentially of dietary origin or obtained by reduction of sphingosine (d18:1), and phytosphingosine (t18:0), which are present in yeast and generated in mammals, are other sphingoid bases that occur in ceramides and have specific roles in cell membranes (Jojima and Kihara, 2023; Mashima et al., 2019; Sugawara, 2022; Su et al., 2017; Uchino et al., 2023; Wan et al., 2019). Thus, a large number of sphingolipids can be altered by fumonisins due to their effects on CerS, but, with the exception of m18:1- and m18:0-ceramides, the effects of fumonisins on sphingolipids other than d18:0- and d18:1-containing ones have not been investigated to date.

The objective of this study was to compare the effects of fumonisins on the sphingoid bases and sphingolipids obtained by de novo synthesis, salvage, and recycling (Fig. 1). The analysis focused on liver and kidney, for which long-chain and very-long-chain ceramides have been reported to vary after fumonisin exposure (Guerre et al., 2022b, 2022c; Tardieu et al., 2021). Plasma was also analysed to investigate systemic changes.
The study was carried out in chickens at 14 and 21 days of exposure for comparison with the effects of fumonisins previously observed at 4 and 9 days of exposure on d18:1, d18:0, m18:0, m18:1, sphingoid bases, and the corresponding sphingolipids. The level of fumonisins in the feed has been chosen to be close to the maximum level of 20 mg FB1 +FB2/kg feed recommended by the European Commission for poultry species (“COMMISSION RECOMMENDATION of 17 August, on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding,” 2006, 2016). The targeted UHPLC-MSMS method that was previously developed for the quantification of sphingolipids was completed by determination of m17:0, m17:1, d18:2, and t18:0, and the corresponding ceramides.

2. Materials and methods

2.1. Analytes and reagents

The analytes and reagents used in this study were obtained from Sharlab (Sharlab S.L., Sentmenat, Spain) or Sigma (Sigma-Aldrich Chimie SARL, Saint Quentin Fallavier, France).

2.2. Feed and animals

Two experimental diets were formulated on a corn-soybean basis by Tecaliman (Tecaliman, 44323 Nantes, France) to best meet the nutritional needs of the chickens. Feed containing fumonisins was made by incorporating naturally contaminated corn to final concentrations of 9.4, 5.21, and 0.83 mg/kg of feed, of FB1, FB2, and FB3, respectively. Control diet was made using the same formula without mycotoxins.

Mycotoxins in the diets were measured by Labocéa (Labocéa, 22440 Ploufragan, France) using LC-MSMS according to AFNOR V03-110 ("ANSES_GuideValidation.pdf," 2015). Except for fumonisins, all mycotoxins assayed were not detected or only present at trace concentrations (Table S1).

The study was carried out at Cebiphar (Cebiphar, 37230 Fontettes, France) in accordance with the European Directive EC2010/63 for the care and use of animals for research purposes under number V11941, project 2017062111426641 accepted by the French Ministry of Higher Education, Research and Innovation on 6 November 2017. One-day-old chicks randomly distributed in six floor pens were housed indoors at Education, Research and Innovation on 6 November 2017. One-day-old project 2017062111426641 accepted by the French Ministry of Higher Education, Research and Innovation on 6 November 2017.

Three experimental diets were formulated on a corn-soybean basis by Tecaliman (Tecaliman, 44323 Nantes, France) to best meet the nutritional needs of the chickens. Feed containing fumonisins was made by incorporating naturally contaminated corn to final concentrations of 9.4, 5.21, and 0.83 mg/kg of feed, of FB1, FB2, and FB3, respectively. Control diet was made using the same formula without mycotoxins.

Mycotoxins in the diets were measured by Labocéa (Labocéa, 22440 Ploufragan, France) using LC-MSMS according to AFNOR V03-110 ("ANSES_GuideValidation.pdf," 2015). Except for fumonisins, all mycotoxins assayed were not detected or only present at trace concentrations (Table S1).

2.3. Biochemistry and UHPLC-MSMS analysis of fumonisins in tissues

Plasma was assayed using a KONELAB 20 clinical chemistry analyser (Fisher Scientific SAS, 67400 Illkirch, France) according to the manufacturer’s instructions. Uric acid, cholesterol, proteins, albumin, globulins, and the activities of lactate dehydrogenase (LDH, EC 1.1.1.27), alkaline phosphatase (ALP, EC 3.1.3.1), alanine aminotransferase, (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), and creatinine phosphokinase (CPK, EC 2.7.3.2) are reported in Table S3.

The concentrations of FB1, FB2, and FB3 in liver and kidney were determined by UHPLC-MSMS analysis as previously described (Tardieu et al., 2019). Briefly, one gram of tissue was homogenized in 2 mL of 0.9% NaCl with an Ultra-Turrax® device, and 2 mL of acetonitrile/methanol (1:1) and 20 µL of a mixture containing the radiolabelled fumonisins was used as IS were added. The samples were placed on a stir table for 2 h and then centrifuged for 15 min at 3000 x g to obtain the supernatant fraction. After lipid extraction with hexane, the aqueous phase was extracted on a FUMONIPREP® column according to the manufacturer’s instructions. The eluant was collected and evaporated to dryness. The UHPLC conditions, multiple reaction monitoring (MRM) parameters, retention times, and validation of the method were described in (Guerr et al., 2022b; Tardieu et al., 2019).

2.4. UHPLC-MSMS analysis of sphingolipids

Extraction of sphingolipids from liver, kidney, and plasma was carried out as previously described (Tardieu et al., 2021). Briefly, 1 g of tissue was homogenized with a Potter-Elvehjem homogenizer in 3 mL of phosphate buffer (0.1 M, pH 7.4) and then centrifuged at 3000 g for 15 min. Forty microlitres of the supernatant was diluted with 120 µL of 0.9% NaCl and spiked with 10 µL of a solution containing the IS to a final concentration equivalent to 6250 pmol/g. Six hundred microlitres of methanol/chloroform (2/1, v/v) were added. After incubation overnight at 48 ºC, 100 µL of 1 M KOH in methanol was added and the samples incubated for 2 h at 37 ºC, followed by the addition of 10 µL of 50% acetic acid. The samples were centrifuged, the supernatant collected, and the residue was extracted again. The two supernatants were pooled, evaporated to dryness, and the dry residue was dissolved in 200 µL of MeOH. A 5 µL aliquot was injected into the chromatographic system after filtration. The sphingolipids in plasma were extracted with the same method using 40 µL of plasma.

Separation of the analytes was carried out on a Poroshell 120 column (3.0 × 50 mm, 2.7 µm) using a 1260 autosampler binary pump from Agilent (Agilent, Santa Clara, CA, USA) as previously described (Tardieu et al., 2021). Detection was performed in dynamic MRM with an Agilent 6410 Triple Quadrupole Spectrometer after positive electrospray ionization at 300 ºC at a flow rate of 10 mL/min under 25 psi and 4000 V capillary voltage. The analytes corresponded to those previously described in (Tardieu et al., 2021), completed by m17:0-, m17:1-, m18:0-, m18:1-, d18:2-, and t18:0-ceramides and the corresponding sphingoid bases. The MRM transitions, fragmentor voltages, and retention times, and validation of the method were described in (Guerr et al., 2022b; Tardieu et al., 2019).
collision energies of all the sphingolipids assayed in this study are reported in Table S2. 4E,8Z-sphingadiene (d18:2[4_8]), that can be formed by the RSD of the recovery measured on the IS, which should be below 20%, is reported in Table S5. The concentrations of sphingolipids not available as standards were calculated using the calibration curves obtained for analytes of a same class with similar mass. The final concentrations in liver, kidney, and plasma were corrected by the recovery measured for the corresponding IS.

2.5. Partial least-square discriminant analysis and long-chain to very-long-chain sphingolipids ratios

Because of the numerous effects of fumonisins on sphingolipids, partial least-square discriminant analyses (PLS-DA) were conducted to reveal the variables that were the most discriminant in separating the chickens into different groups. Two strategies of analysis were used. In the first one, the chickens were divided into three groups according to the duration of fumonisin feeding. In the second one, the chickens were divided into two groups according to the presence or absence of fumonisins in the feed. The variables that had a score above 1.3 were selected to highlight the most important sphingolipids in the repartitions of chickens into the different groups.

Because numerous sphingolipids of different classes were affected by fumonisins, and because the effects of fumonisins on the total sphingolipids amount in a same class varied with the organ studied, ratios were calculated to compare the relative proportions of long-chain and very-long-chain sphingolipids. CerS2 is known to form C22-C26 ceramides and is highly expressed in liver and kidney, while CerS5 in liver, and CerS5 and CerS6 in kidney, are both involved in the formation of C14-C16 ceramides (Cingolani et al., 2016; Mullen et al., 2012). Consequently, the ratios between the sum of C22 to C26 and the sum of C14 to C16 sphingolipids were calculated. Because of the low abundance of C14-, C15-, C25-, and C26-sphingolipids, compared to the abundance of C16-, C22-, C23-, and C24-sphingolipids, the C22:C24:C16 ratios were also calculated. Because the ratio varied greatly in the controls according to the class of ceramide, the results are expressed in fold-change to permit a better comparison of the effects of fumonisins.

2.6. Statistical analysis

Statistical analyses were performed using XLSTAT Biomed software (Addinsoft, 33000 Bordeaux, France). All results are reported as means ± SD, differences among groups were considered significant for P < 0.05. Correlations among the sphingolipids bases were investigated by determination of Pearson’s coefficient. The effects of fumonisins on the sphingolipidome were first assessed globally using partial least square discriminant analysis (PLS-DA). Three analysis strategies were conducted. In the first analysis, chickens not exposed to fumonisins receiving the control diet (n = 10) were compared with chickens receiving the diet containing fumonisins (n = 20). The Q² values obtained for liver, kidney, and plasma for the first two components were 0.867, 0.959 and 0.806, respectively. In a second analysis, chickens fed the control diet (n = 10) were compared with chickens fed the diet containing fumonisins for 14 (n = 10) or 21 days (n = 10). The Q² values obtained for liver, kidney and plasma for the first two components were 0.649, 0.698 and 0.482, respectively. In a third analysis, chickens fed the control diet that were sacrificed on day 35 (n = 5), and chickens fed the control diet that were sacrificed on day 36 (n = 5), were compared with chickens fed the diet containing fumonisins for 14 days that were sacrificed on day 36 (n = 10), and chickens fed the diet containing fumonisins for 21 days that were sacrificed on day 35 (n = 10). The Q² values obtained for liver, kidney and plasma for the first two components were 0.384, 0.426 and 0.343, respectively. Taken together, these analyses indicate that the presence or absence of fumonisins in the diet is the main discriminator for the amount of sphingolipids in liver, kidney and plasma. The duration of exposure to fumonisins also discriminated sphingolipid content in liver and kidney, but the robustness of the model was poor in plasma. The day of slaughter did not appear to be discriminatory, regardless of the matrix analysed. It was therefore decided to present the results of analysis 2 in the manuscript, with the results of analysis 1 reported in Supplementary material (Fig. S2). The variables measured within the chickens fed the control diet (n = 10), and the chickens fed the diet containing fumonisins for 14 (n = 10) or 21 days (n = 10) were then compared using one-way ANOVA after testing for homogeneity of variance (Hartley’s test). When a significant difference was observed, individual means were compared (Duncan). Different letters in the same row indicate statistically different means.

3. Results and discussion

3.1. Health impact and fumonisins in tissues

Administration of fumonisins at a dose of 14.6 mg of FB1 + FB2/kg of feed for 14 and 21 days to growing chickens resulted in no effects on feed consumption, animal and organ weights, or biochemical parameters (Table S3). This is in agreement with numerous other studies, which have led to assignment of the maximum tolerable level of fumonisins in chicken feed as 20 mg FB1 + FB2/kg (EFSA et al., 2018; “FDA, 2011,” 2016). Recent studies have suggested that 2.5 mg FB1 + FB2/kg of feed for 21 days has an effect on the size of the intestinal crypts and performances, but no reassessment of the maximum tolerable level of fumonisins in animal feed has been carried out to date (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2022; Sousa et al., 2020).

Fumonisin B1 accumulated in the liver of chickens with increasing duration of exposure (Table 1). The concentrations measured are consistent with those previously described in this organ (Hort et al., 2020; Laurain et al., 2021; Tardieu et al., 2021a; Tardieu et al., 2019). The FB1 concentrations in kidney were lower than those found in liver, and similar at 14 and 21 days of exposure. FB1 has previously been found to increase between 4 and 9 days in kidney in chickens (Guerre et al., 2022b). All these results confirmed a higher accumulation of FB1 in the liver than in the kidney in chickens, contrary to what has been described in rats (Riley and Voss, 2006).

3.2. Sphingolipids according to their sphingoid bases in chickens

Sphinganine (Sa = d18:0), sphingosine (So = d18:1), m18:0, m18:1, and t18:0 were detected in liver and kidney, while m17:0, m17:1, and d18:2 were not detected or only present at traces levels (Table 1). The second sphingoid base in abundance assayed in liver and kidney was
t18:0, which is in agreement with data in mammals (Sugawara, 2022), Fumonisins, sphingoid bases and their derivate in liver, kidneys and plasma of chickens fed a control diet free of mycotoxins and chickens fed for 14 or 21 days with an intake containing 9.4 and 5.21 mg/kg of FB1 and FB2, respectively.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Control</th>
<th>Liver</th>
<th>Kidney</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1^2</td>
<td>ND</td>
<td>22 ± 4.8^b</td>
<td>28.7 ± 5.4^a</td>
<td>ND</td>
</tr>
<tr>
<td>FB2</td>
<td></td>
<td>3.4 ± 0.6</td>
<td>3.5 ± 0.9</td>
<td>ND</td>
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<tr>
<td>d18:0^a</td>
<td>2.3 ± 0.6x</td>
<td>7.3 ± 2.4x</td>
<td>13.2 ± 5.3</td>
<td>0.3 ± 0.1^b</td>
</tr>
<tr>
<td>d18:1^a</td>
<td>20.3 ± 3.6x</td>
<td>24.6 ± 3.5x</td>
<td>26.4 ± 4x</td>
<td>6.2 ± 1.3^b</td>
</tr>
<tr>
<td>m18:0</td>
<td>17.1 ± 5.1</td>
<td>19 ± 7.7</td>
<td>21.4 ± 4.8</td>
<td>6.6 ± 1.6^b</td>
</tr>
<tr>
<td>m18:1^c</td>
<td>2.1 ± 0.4</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.4</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>t18:0</td>
<td>3.9 ± 0.9</td>
<td>4.8 ± 1.6</td>
<td>4.1 ± 0.9</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>d18:0^b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>d18:1^b</td>
<td>151 ± 24^b</td>
<td>210 ± 111</td>
<td>320 ± 85</td>
<td>279 ± 86</td>
</tr>
<tr>
<td>18:0/20^2</td>
<td>57 ± 14.7^x</td>
<td>146 ± 45</td>
<td>187 ± 46^x</td>
<td>18.6 ± 2.8^x</td>
</tr>
<tr>
<td>18:1/20^3</td>
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<td>0.3 ± 0.1^c</td>
<td>0.3 ± 0.1^c</td>
<td>79.6 ± 22^b</td>
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<tr>
<td>Gloco^d</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0</td>
<td>33.9 ± 10.2</td>
<td>32 ± 9.3</td>
</tr>
<tr>
<td>LysoSM^e</td>
<td>139 ± 18^b</td>
<td>164 ± 41</td>
<td>182 ± 32</td>
<td>267 ± 52</td>
</tr>
<tr>
<td>ScSo</td>
<td>0.11 ± 0.03c</td>
<td>0.30 ± 0.11b</td>
<td>0.49 ± 0.15a</td>
<td>0.05 ± 0.01b</td>
</tr>
<tr>
<td>Sa1P</td>
<td>Sa1P</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, n = 10. 1 in nmol/g of tissue or in nmol/mL of plasma and 2 in pmol/g of tissue or in pmol/mL of plasma. d18:2, m17:0, m17:1, and LacSo were not detected in this study or only present at the trace level (ND). ANOVA was used to assess the difference between groups. Statistically different groups (Duncan) are identified by different letters (p < 0.05).

Table 2

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Liver</th>
<th>Kidney</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>m17:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>m17:1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>m18:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>m18:1</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>m18:2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>m18:3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>m18:4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<td>m18:6</td>
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<tr>
<td>m19:3</td>
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</tbody>
</table>

Results are expressed as mean ± SD, n = 10. 1 in nmol/g of tissue or in nmol/mL of plasma and 2 in pmol/g of tissue or in pmol/mL of plasma. d18:2, m17:0, m17:1, and LacSo were not detected in this study or only present at the trace level (ND). ANOVA was used to assess the difference between groups. Statistically different groups (Duncan) are identified by different letters (p < 0.05).
10% d18:0- and m18:1-ceramides, 15–20% d18:2- and d18:1-ceramides, and 50% t18:0-ceramides in liver and kidney (Fig. 2).

In plasma, the diversity of ceramides was less, but their proportions also varied. High ceramide concentration variability depending on the sphingoid base, the fatty acid, and the tissue analysed is in agreement with previous studies highlighting the complexity of sphingolipids metabolism in cells (Mashima et al., 2019; Merrill, 2011; Sugawara, 2022; Wan et al., 2019).

3.3. Effects of fumonisins on the sphingoid bases

Administration of 14.6 mg FB1 + FB2/kg feed for 14 and 21 days increased the sphinganine levels in liver, kidney, and plasma (Table 1). Sphingosine was also increased in liver, kidney, and plasma, and the Sa:So ratio increased in the three matrices assayed, but the effect was not significant in plasma. A small significant decrease in m18:0 was observed in the kidneys of fumonisin-fed animals, while no significant effect was observed in the liver. No significant effect of feeding fumonisins was observed on m18:1 and t18:0 in any matrix. Sphingosine 1-phosphate (So1P = d18:1 P) increased in liver, was unaffected in kidney, and decreased in plasma.

Sphinganine 1-phosphate (Sa1P = d18:0 P) was only detected in plasma and was increased by feeding fumonisins. The Sa1P:So1P ratio can only be measured in plasma and was significantly increased by feeding fumonisins, with the effect being more pronounced at 21 days than at 14 days. The concentrations of N-acetylsphinganine (18:0/2:0) and N-acetylsphingosine (18:1/2:0) in liver and kidney were also increased by fumonisins, while that of 18:1:2:0 was unaffected and 18:0/2:0 was not detected in plasma. Interestingly, most of the sphingoid bases in liver and kidney correlated together, with the exception of phytosphingosine (t18:0) (Fig. 3).

The effects of fumonisins on d18:0, d18:1, m18:0, and m18:1 and their derivatives measured at 14 and 21 days of exposure in liver and kidney are in agreement with previous results in chicken at 9 days of exposure (Guerre et al., 2022b; Tardieu et al., 2021). This study is the first to report t18:0 in animals fed fumonisins, and it was unaffected by the mycotoxin.

3.4. Effects of fumonisins on d18:0- and d18:1-sphingolipids

A decrease in the total ceramides concentration was observed in the livers of chicks fed fumonisins, while no significant effect was observed in the kidneys (Table 3). Additionally, a significant decrease in most of the ceramides assayed, with the exception of C24- to C26-ceramides, occurred in the liver. Concerning the kidneys, most of the C14- to C18 ceramides decreased, while most of the C22- to C26 ceramides increased. The effects of fumonisins on dihydroceramides (DHCer) were similar to those observed for ceramides: 18:0/16:0 decreased or tended to decrease in liver and kidney, while 18:0/24:0 increased or tended to increase. The effects of fumonisins on other DHCer in liver and kidney were generally not significant. In plasma, the only significant effect of fumonisins on d18:0- and d18:1-ceramides was a decrease in 18:1/16:0 (Table 3).
Table 3
d18:1-and d18:0-sphingolipids in liver, kidneys and plasma of chickens fed a control diet free of mycotoxins and chickens fed for 14 or 21 days with a diet containing 14.6 mg FB1 + FB2/kg.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 14 d</td>
<td>FB 14 d</td>
<td>Control 21 d</td>
</tr>
<tr>
<td><strong>Ceramides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1/23:0</td>
<td>51.6 ± 8.6</td>
<td>51.6 ± 7.9</td>
<td>51.6 ± 7.9</td>
</tr>
<tr>
<td>18:0/22:0</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>18:0/18:0</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
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<tr>
<td>18:1/24:1</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
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<tr>
<td><strong>Dihydroceramides</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18:1/25:1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>18:0/22:0</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
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<tr>
<td>18:0/18:0</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
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<tr>
<td>18:1/24:1</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
</tr>
<tr>
<td><strong>Sphingomyelins</strong></td>
<td></td>
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<tr>
<td>18:1/25:1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>18:0/22:0</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
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<tr>
<td>18:0/18:0</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
</tr>
<tr>
<td>18:1/24:1</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
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<tr>
<td><strong>Monoceramides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1/25:1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>18:0/22:0</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
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<tr>
<td>18:0/18:0</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
<td>21.8 ± 1.5</td>
</tr>
<tr>
<td>18:1/24:1</td>
<td>227 ± 4.6</td>
<td>227 ± 4.6</td>
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(continued on next page)
The total sphingomyelin concentration was increased in the liver and kidney of chickens fed fumonisins (Table 3). Most of the sphingomyelins assayed were increased in the liver, while, in the kidneys, C14- to C17-sphingomyelins decreased and C22- to C26-sphingomyelins increased. Total dihydrosphingomyelins (DHSM) were increased in the liver but not in the kidneys of chickens fed fumonisins (Table 3). At the analyte level, most of the DHSM assayed in the liver were increased. SM18:0/22:0, SM18:0/24:0, and SM18:0/24:1 were also increased in the kidney. The effects of fumonisins on sphingomyelins and DHSM in plasma were close to those observed in kidney, but less pronounced.

The total concentrations of monohexosylceramides (HexCer) and the total concentrations of lactosylceramides (LacCer) were unaffected by feeding fumonisins in liver and kidney, but were decreased in plasma (Table 3). At the analyte level, HexC18:1/16:0 and LacC18:1/16:0 were decreased in liver, kidney, and plasma of chickens fed fumonisins. The effects of fumonisins on the other glycosylceramides were generally weak and varied according to the compound and organ studied.

3.5. Effects of fumonisins on m17:0, m17:1, m18:0, m18:1, d18:2, and t18:0-ceramides

The only ceramide containing m17:1 found in this study was m17:1/24:1, and feeding fumonisins had only a weak effect on its concentration (Table 2). The total concentrations of m18:0-, m18:1-, d18:2-, and t18:0-ceramides decreased in the liver of fumonisin-fed chickens, but were unaffected in kidney, with the exception of m18:1 ceramides, which decreased (Table 2). At the compound level, most of the ceramides were decreased in the liver whereas the effects in the kidney appeared to be less pronounced. Finally, numerous effects of fumonisins on m18:0-, m18:1-, d18:2-, and t18:0-ceramides were observed and it was difficult to tell whether these effects were different from those observed for d18:0- and d18:1-ceramides.

3.6. Overall effects of fumonisins on sphingolipids

3.6.1. Repartitions of chickens into different groups according to the feeding of fumonisins

Partial least-squares discriminant analyses (PLS-DA) were conducted to reveal which sphingolipids were the most discriminating in explaining the repartition of chickens into different groups according to the feeding of fumonisins. PLS-DA of sphingolipids assayed in liver and kidney according to the duration of feeding fumonisins and according to the presence or absence of fumonisins in feed, are presented in Fig. 4 and S2, respectively. Both analyses showed good separation of chickens into the different groups, with good quality of the models used. The variables important in the projection were close in these two models and close in liver and kidney. They corresponded to Sa (d18:1), N-acetylated forms of Sa and So, more So (d18:1) in kidney. Long-chain ceramides 18:1/14:0, 18:1/16:0, and 18:1/17:0, long-chain C15 to C17 sphingomyelins, very-long-chain sphingomyelins, and to a lesser extend very-long-chain ceramides were also important, while only a small number of dihydro sphingomyelins, dihydroceramides, and glycosylceramides were important in the projection (Fig. 4 and S2). Interestingly, m18:1/16:0 and t18:0/24:1 in liver, and 18:2/16:0 in kidney, were the only ceramides not containing d18:1 or d18:0 that appeared to be important in the projection in the two models.

PLS-DA conducted on the sphingolipids assayed in plasma of chickens fed fumonisins are reported in Fig. 5. A good separation of chickens into the groups and a good quality of the model were found irrespective of the analysis strategy used. Interestingly, Sa and So were important variables in the analysis performed using three groups only. In contrast, So1P and Sa1P were important in the projection irrespective of the analysis strategy used. This result is consistent with ANOVA (Table 1) and previous results suggesting that Sa1P:So1P is a better biomarker of fumonisins exposure in plasma than Sa:So (Cai et al., 2007; Castegnaro et al., 1998; Riley et al., 2015a, 2015b; Tardieu et al., 2021; Tran et al., 2006).

The only ceramide important in the projection in the two analyses was 18:1/16:0. Sphingomyelins, notably long-chain C14 to C17 sphingomyelins, were the most numerous variables important in the projection in plasma. Very-long-chain dihydro sphingomyelins SM18:0/22:0, SM18:0/24:1, and SM18:0/24:0, monohexosylceramides HexCer18:1/16:0 and HexCer18:1/24:1, and lactosylceramides LacCer18:1/16:0 were important in the projection in plasma, while none of the m18:0-, m18:1-, d18:2-, and t18:0-ceramides assayed were important.

3.6.2. Long-chain to very-long-chain sphingolipid ratios in controls and fumonisin-fed chickens

Long-chain to very-long-chain sphingolipids ratios were calculated and expressed as fold-changes to allow comparison of the effects of fumonisins according to the class of sphingolipid assayed and the organ studied. Both the C22-C26:C14-C16- and C22-C24:C16-ratios were determined to reveal differential effects of fumonisins according to the Cer5 chain length specificity. C22 to C26 ceramides are formed by CerS5, which is widely expressed in liver, while C14 to C16 ceramides are formed by CerS5 in liver, and CerS5 and CerS6 are formed in kidney (Cingolani et al., 2016; Ho et al., 2022; Mullen et al., 2012). Similar effects of fumonisins on C22-C26:C14-C16 ratios, reported in Fig. S3, and C22-C24:C16 ratios, reported in Fig. 5, were observed. This is consistent with the specificity of CerS5 and the low abundance of C25-, C26-, C14-, and C15-sphingolipids compared to the abundance of C22-, C23-, C24-, and C16-sphingolipids (Table 3). Because C22-, C23-, C24-, and C16-sphingolipids are commonly investigated in metabolomics studies whereas C25-, C26-, C14-, and C15-sphingolipids are not, the C22-C24:C16 ratio is preferable for future between-studies comparison.

A significant increase in the C22-C24:C16 ratio was observed for most of the sphingolipids assayed, with the exception of t18:0-ceramides in liver and kidney, d18:2-ceramides in liver, and d18:1-ceramides in plasma. The lack of a significant effect of fumonisins on the C22-C24: C16 ratio measured on t18:0-ceramides in liver and kidney can be attributed to the low effect of fumonisins on this class of sphingolipids. This hypothesis is consistent with data presented in Fig. 3 that revealed...
Fig. 4. PLS-DA of sphingolipids in liver and kidney of chickens fed a control diet free of mycotoxins and chickens fed a diet containing 14.6 mg FB1 + FB2/kg for 14 or 21 days.
Fig. 5. PLS-DA of sphingolipids in plasma of chickens fed a control diet free of mycotoxins and chickens fed a diet containing 14.6 mg FB1 + FB2/kg for 14 or 21 days.
no correlation between t18:0 and other sphingoid bases. It is also consistent with PLS-DA, which revealed that t18:0/24:1 in liver was the only t18:0-containing sphingolipid important in the repartition of chickens in the different groups (Figs. 4 and 5). The lack of a significant effect of fumonisins on the C22-C24:C16 ratio measured for d18:1-ceramides in plasma can be explained by the relatively low abundance of ceramides in plasma compared to liver and kidney. Interestingly, comparison of the C22-C24:C16 ratio showed that the effects of fumonisins were similar in liver and kidneys, whereas the effects of fumonisins on the total amount of the different classes of sphingolipids varied with the sphingolipids class and the organ studied (Table 3).

### 3.7. Overall discussion of the effects of fumonisins

The main purpose of this study was to compare the effect of fumonisins on different classes of sphingolipids in liver and kidney, and to determine whether systemic effects can be observed in plasma. Thousands of sphingolipids have been identified, all of which are metabolically interconnected and in equilibrium within cells (Merrill, 2011; Sugawara, 2022). Targeted UHPLC-MSMS analysis permits measurement of a large number of sphingolipids, and it allows quantitation of low-abundance analytes in cells for which an isotopic mass overlap with analytes of neighbouring mass, sometimes 100 times more abundant, may interfere in their determination (Merrill, 2011; Wang et al., 2014). m17:0, m17:1, d18:2, and t18:0 sphingoid bases and their ceramides, which have not been investigated to date in animals fed fumonisins, and d18:0–, d18:1–, m18:0–, and m18:1-sphingolipids, for which selective effects of fumonisins have been already characterized in chickens, were selected to be representative of the different pathways of sphingolipids synthesis (Fig. 1) (Mashima et al., 2019; Merrill, 2011; Wan et al., 2019). Strong correlations were observed among m18:0, m18:1, d18:0, d18:1, and d18:2, and the changes for all the ceramides assayed were similar, with the exception of t18:0 and the corresponding ceramides, which appeared to be relatively less sensitive to fumonisins. PLS-DA revealed that most of the m17:1-, m18:0-, m18:1-, d18:2-, and t18:0-ceramides assayed were not important variables for explaining the repartition of chickens into different groups according to the feeding of fumonisins. In contrast, d18:0 and d18:1, their acetylated and phosphorylated derivatives, long-chain d18:1-ceramides, and long-chain and very-long-chain d18:1-sphingomyelins were important variables in these analyses, which is in agreement with previous results in chickens and turkeys (Guerre et al., 2022b, 2022c; Tardieu et al., 2021).

Measurement of the C22-C24:C16 ratios allows comparison of the selective effects of fumonisins on ceramides according to their fatty acid chain length in the various organs studied. This analysis revealed that the effects of fumonisins on d18:0-, m18:1-, and d18:2-ceramides were similar to those observed for d18:1-ceramides, and no effect was observed for t18:0-ceramides. Moreover, it is interesting to highlight that the effects of fumonisins on C22-C24:C16 ceramide ratios were similar even though the total amount of ceramides decreased in the liver.
but was unaffected in the kidneys. A decrease in the total ceramides contents in the liver due to the inhibition of CerS is the expected effect of fumonisins (Riley and Merrill, 2019), but a previous study at 9 days of exposure in chickens revealed that the total ceramides content was unaffected by fumonisins (Tardieu et al., 2021). Differences between studies can be explained by the duration of exposure, which was longer in this study, allowing for a decrease in the total ceramides to become established. The lack of a decrease in the total ceramides content of the kidney agrees with previous results at 4 and 9 days of exposure in chickens (Guerre et al., 2022b). This was explained by a “compensatory increase” occurring for very-long-chain ceramides secondary to the decrease of long-chain ceramides, which is a mechanism that has already been observed in CerS knockout mice and non-alcoholic fatty liver disease (Park et al., 2014; Pewzner-Jung et al., 2010b, 2010a). Differences between liver and kidneys can be explained by the lowest concentration of fumonisins present in kidneys compared to liver. Consequently, all the studies conducted to date in chickens and turkey fed fumonisins revealed that the effects of fumonisins on ceramides differed according to the CerS involved in their synthesis (Guerre et al., 2022c, 2022d; Tardieu et al., 2021). Variation of sphingolipid concentrations according to their fatty acid chain length have also been reported in pigs fed fumonisins (Loiseau et al., 2015). A decrease in C20-, C22-, and C24-ceramides was observed in pig lung, while no effect was found in the liver. Because pulmonary oedema is a key disease in pigs fed fumonisins, the ceramide changes observed were considered to play a role in the toxicity (Loiseau et al., 2015). In mice, the administration of 10 mg FB1/kg BW for 21 days caused a significant decrease in C16-ceramides and C16-sphingomyelins, while little effect was observed on C22-C24-ceramides and C22-C24-sphingomyelins, and no signs of hepatotoxicity occurred. When administered to mice on a high-fat diet, FB1 exacerbated hepatotoxicity and led to a reduction in all ceramides and sphingomyelins, with the data presented suggesting that the effect was more pronounced on very-long-chain sphingolipids than on long-chain ones (Dopavogui et al., 2023). Changes in the long-chain to very-long-chain ceramides and sphingomyelin ratios have now been reported in different diseases in humans, independently of the ingestion of fumonisins, and are considered to be a key issue for the toxicity (Aslan et al., 2023; Ho et al., 2022; Höring et al., 2022; Liang et al., 2023; Montefusco et al., 2022; Westhölt et al., 2022). Although the brain contains high levels of sphingolipids and is a target organ for fumonisins, its analysis was not included in this study. Previous work in chickens showed that FB1 was not detectable in this organ and that, although significant changes in the sphingolipidome were observed, they did not appear to correspond to those observed in the liver and kidney and therefore warranted further specific studies (Guerre et al., 2022b).

The decreases in total ceramide content measured in this study after 14 and 21 days of exposure were observed in the liver at concentrations of 0.022 and 0.029 µmol FB1/kg, respectively. No effect of fumonisins on total ceramide content was observed in the kidney, where 0.014 and 0.011 µmol FB1/kg were found after 14 and 21 days of exposure, respectively. These results seem to be in agreement with the IC50 of 0.1 µmol FB1/L measured for ceramide synthases in rat hepatocytes in culture (Wang et al., 1991). Although the IC50 of FB1 on glycosylceramide synthesis in cell culture is higher than the IC50 measured for ceramides (Merrill et al., 1993), the changes in C22-C24/C16 glycosylceramide ratios observed in this study are consistent with the schematic of their synthesis (Fig. 1). The relatively long exposure of chickens to fumonisins compared to the cell culture probably allowed the decrease in d18:0-Cer, d18:1-Cer, d18:0-SM, d18:1-SM, and t18:1-Cer to become established. The effects of fumonisins on C22-C24:C16 sphingomyelins ratios were similar to those observed for ceramides, but involved an increase in total sphingomyelin content in liver and kidney, which is in agreement with previous results in chickens and turkeys (Guerre et al., 2022b, 2022c; Tardieu et al., 2021). The increase in total sphingomyelins is difficult to explain as a decrease, secondary to a decrease in ceramides synthesis and possible hydrolysis of sphingomyelins from the membrane cells, was expected (Fig. 1) (Riley and Merrill, 2019). Although no explanation can be provided, an increase in sphingomyelin content has already been reported in cells after selective knockdown of ceramide synthases, revealing that the ceramide/sphingomyelin rheostat is very complex (Mullen et al., 2011; Tani-guchi and Okazaki, 2020). The lack of a decrease in sphingomyelins concentrations is probably important in explaining the relative tolerance of chickens and turkeys toward fumonisins. Indeed, hydrolysis of sphingomyelins precedes ceramide-induced apoptosis, which has been reported to occur at high doses of fumonisins (Burger et al., 2018; Lumsangkul et al., 2021; Mignard et al., 2020; Riedel et al., 2016; Song et al., 2021). Although the origin of the increase in sphingomyelin content in this study remains unknown, and the involvement of an unidentified confounding factor cannot be completely ruled out, the increase in sphingomyelin content does not prevent an increase in the C22-C24: C16 ratio observed for d18:0-Cer, d18:1-Cer, HexCer, LacCer, d18:0-SM, d18:1-SM, and t18:1-Cer in liver and kidney. Finally, it should be highlighted that changes in the C22-C24:C16 sphingolipid ratios were also observed in plasma. Although the mechanisms behind these effects are not yet understood, this last result suggests that C22-C24:C16 sphingolipids ratios, in conjunction with the Sa1P:So1P ratio, could be good candidates for revealing fumonisin exposure in chicken plasma at doses that had no effect on the Sa:So ratio.

4. Conclusions

In conclusion, this study revealed that the effects of fumonisins on the total amount of sphingolipids varied according to the class of sphingolipid assayed and the organ studied. The effects on d18:0 and d18:1-sphingolipids were the most pronounced, with the changes in m17:1, m18:0, m18:1, d18:2, and t18:0 and the corresponding ceramides being less substantial and of limited value in explaining the partition of chickens into different groups according to the feeding of fumonisins. The C22-C24:C16 ratios were increased irrespective of the class of sphingolipid assayed or the organ studied, with the exception of t18:0-ceramides, which were unaffected. Altogether, the effect of fumonisins on the C22-C24:C16 ratio and the lack of a decrease in the sphingomyelin contents in liver and kidney are probably key points in explaining the relative tolerance of chickens to fumonisins. Further studies are necessary to investigate the possible use of C22-C24:C16 ratios as a biomarker of fumonisin exposure.

CRediT authorship contribution statement

Philippe Guerre: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing.
Elodie Lassallette: Formal analysis, Writing – original draft. Pi Nyvall Collen: Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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