Effect of fumonisins and *Salmonella* on digestive flora profiles assessed using a molecular tool (CE-SSCP).

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Abstract

Fumonisins (FB) are mycotoxins frequently found in vegetal feedstuffs, especially in maize used for pig feeding. Among fumonisins, FB₁ was the better described toxin. It caused pulmonary and hepatic damages as well as immune response disorders in pigs that were recognised as especially sensitive to FB intoxication. The FB₁ immunosuppressor induced a higher susceptibility of pigs to gut pathogens such as *E coli*. Effects on *Salmonella* have poorly been studied despite the frequent asymptomatic carriage in pigs and the presumptive role of flora equilibrium on prevention of *Salmonella* excretion or re-excretion. To determine the influence of *Salmonella* carriage, fumonisins or both on digestive flora equilibrium, the use of a molecular technique: CE-SSCP (Capillary-E lectrophoresis Single Strand Conformation Polymorphism) appeared a good complement to the conventional bacteriological techniques. The objective was to assess the perturbation of flora associated with co-exposition in experimental conditions in absence of clinical sign.

Forty eight piglets were clustered following a 2x2 "factorial scheme" in order to analyse on faecal flora, the effect of a feeding naturally contaminated with FB (8.5 ppm of FB₁ and 2.8 ppm of FB₂) associated with an asymptomatic carriage of *Salmonella Typhimurium*. The effect of FB and *Salmonella* has been investigated onto 10 week old piglets and during 9 weeks. Faeces of the pigs were taken regularly. Bacteriological numeration of total aerobic flora was conducted. DNA of each sample was extracted using the QI Amp DNA Stool Minikit. The extracted DNA were pooled, the PCR amplification of the rDNA 16S V3 region was carried out. Then the PCR products were analysed by CE-SSCP. Profiles were classified via dendrograms using the BioNumerics software and the Jaccard coefficient for similarity determination.

In this study, 5.10⁶ CFU *Salmonella* per pig induced infections and asymptomatic carriages that didn't affect the faecal flora profiles. Intoxication of the pigs by the contaminated feed has been confirmed by the increase of the sphinganine/sphingosine ratio. The 8.5 ppm concentration of FB₁ did not induce any effect on the animal health indicators, but it affected transiently the digestive flora equilibrium. In case of co-infection with FB and *Salmonella*, the flora profiles were rapidly and strongly modified as soon as 48h post *Salmonella* infection. Therefore under our experimental conditions, exposure to a medium concentration of FB in naturally contaminated food had no effect on the pig health but can affected the digestive flora equilibrium, the *Salmonella* exposure amplifying this phenomenon.

Introduction

The contamination of pig herds by the ubiquitous *Salmonella* may lead to subclinical infection in pigs (Humbert, 1997). Then contaminations of carcasses by this pathogen constitute a threat to human health. Ingestion of contaminated pork meat may lead to food-borne illness. Therefore, decrease of the *Salmonella* contamination level throughout food-chain appeared to be crucial for human health (Giovannacci, et al., 2001). Epidemiological studies identified risk factors associated with *Salmonella* excretion (Beloeil, et al., 2004): implementation of hygiene measures and preservation of digestive ecology equilibrium (Fravalo, et al., 2002) would reduce *Salmonella* prevalence.

Fumonisins (FB) are mycotoxins, secondary metabolites of fungi (*Fusarium moniliforme* and *Fusarium proliferatum*), which may contaminate animal and human feeds. Their global occurrence
is considered as an important risk for human and animal health, as up to 25% of the world crops production may be contaminated with mycotoxins. In pig, a chronic exposure to FB1 are associated with alteration of sphingolipid metabolism and hepatotoxicity. In this specie, FB1 is a predisposing factor for gut infectious disease by local immunosuppressor effect (Oswald, et al., 2003).

The impact of Fumonisins contaminated feed on salmonella excretion intensity in a herd containing asymptomatic carrier pigs has, until now, never been investigated. To determine the influence of Salmonella, fumonisins or both on digestive flora equilibrium, the use of a molecular technique of CE-SSCP (Capillary-E lectrophoresis Single Strand Conformation Polymorphism) appeared a good complement to the conventional bacteriological techniques (Tanguy, et al., 2007). The objective of the present study was to assess the perturbation of noro of SPF pig’s groups in relation to co­ exposition (FB or/and Salmonella) in experimental conditions.

Material and methods
Forty eight piglets were clustered following a 2x2 “factorial scheme” after randomisation the day of weaning:

- 12 piglets FB(-)-Salmo(-)
- 12 piglets FB(+)–Salmo(-)
- 12 piglets FB(-)-Salmo(+)
- 12 piglets FB(+)–Salmo(+)

FB(+)-Salmo (+) and FB(+)–Salmo (+) piglets fed a diet containing 8.5 ppm FB1 and 2.8 ppm FB2 (incorporation of 15% of naturally contaminated maize) since week 7 of age. FB(-)-Salmo(+)and FB(+)-Salmo(+) piglets were inoculated at 8 weeks of age by 5.10^5 UFC Salmonella /pig.

Sa/So determination
Free sphinganine and free sphingosine were determined in pig serum, liver, and kidney by HPLC according to Riley et al. (1993) with minor modifications (Riley, et al., 1993;Tran, et al., 2003). Determination of Sa/So ratio is used to test the intoxication of the pigs following the ingestion of naturally contaminated food.

Salmonella enumeration
The detection and quantitative asessement for Salmonella were carried out in the faeces according to the methods described by Fravalo (Fravalo, et al., 2003a;Fravalo, et al., 2003b).

Total aerobic enumeration
Fecal bacteriological numeration (total aerobic flora) was conducted for each pig at each sampling date by dilution plating on PCA ( incubated 48h/30°C).

DNA extraction/amplification
Fecal samples DNA extractions were performed using a QIAamp DNA Stool Minikit (Qiagen) (McOrist, et al., 2002). One grams of fresh feces were homogeneised with 7 mL of lysis buffer, then 1.6 mL suspensions were used for DNA extraction. DNA coding for the V3 region of the 16S rDNA was amplified from 1 µL of the DNA solution, using the w49 (AGGTCCAGACTCCTACGGG)-w104* (TTACCGGCGGTGCTGGCAC) primer couple. PCR conditions were as follows: 2' at 94°C, and then 25 cycles of 30' at 94°C, 30' at 61°C, 30' at 72°C and a final elongation of 10' at 72°C.

Migration of PCR products
CE-SSCP consisted in the migration of DNA single strands into the 50-cm capillaries of the four­ capillary AbiPrism Genetic Analyser 3100 Avent sequencer (Applied Biosystems, France). After a 1:5 dilution of the amplification products obtained, one µL was dispensed per well and a mixture of 18.5 µl formamide (Applied Biosystems) and 0.5 µl of Genescan-standard-HD-400-rox internal Standard (Applied Biosystems) were then added. The amplification products were heat-denatured (5 minutes/90°C), then cooled for ten minutes in ice added with water. Migrations took place in a polymer (6.22 g CAP polymer (Applied Biosystems), 1 g Glycerol (In Vitrogen), 1 mL 10X buffer (Applied Biosystems) and water for injection (Cooper) q.s. 10 mL) at 32°C under a power of 15 kV.

Result analysis
Profile analyses were performed with the GeneMapper (Applied Biosystems) and Bionumerics (Applied Maths) software. The GeneMapper software was used to align the profiles obtained based on the migration internal standard. Similarity of the profiles was studied by analyzing the presence/absence of bands from profile to profile using Bionumerics software. The Jaccard index (Legendre and Legendre, 1998) was selected during this project for studying profile similarity.
Results

The chronic effect of the FB1 on the Sa/So ratio after the 9 weeks of treatment is presented in Table 1. A significant increase of the Sa/So ratio, in the kidney, the liver, or the serum, was observed during the test, comparing the batches fed with FB1 contaminated food with the control batches.

<table>
<thead>
<tr>
<th>Day before or after inoculation</th>
<th>Kidney</th>
<th>Liver</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-5</td>
<td>ND</td>
<td>ND</td>
<td>+6%</td>
</tr>
<tr>
<td>D+2</td>
<td>+19%*</td>
<td>+50%*</td>
<td>+45%*</td>
</tr>
<tr>
<td>D+56</td>
<td>+54%*</td>
<td>+60%*</td>
<td>+149%*</td>
</tr>
</tbody>
</table>

*significant increases compared with the control animals (batches FB1(-)) (ANOVA and test of Tuckey)

Table 1 - Sa/So ratio increase in the FB1 exposed pigs batches (FB1(+) Salmo(- or +)) compared to non exposed batches (FB1(-) Salmo(- or +)) in the kidney, the liver, and the serum, according to time.

The Salmonella inoculation conducted to a contaminated batch, the majority of the pigs shedding Salmonella (Table 2). The proportion of shedding pigs did not vary during the test depending on FB1 presence. Nevertheless, only 3 of the 12 pigs had a countable excretion in batch FB1(-) Salmo(+) against 9 out of 12 in the batch FB1(+) Salmo(+) two days post inoculation, but this difference is not significant and disappeared as of 7 days post inoculation. Salmonella was countable on 75-100% of the excretory pigs along the growth period.

Table 2 Search for Salmonella Typhimurium in faeces of pigs of the batches FB(-) Salmo(+) and FB(+) Salmo(+) during 49 days after the inoculation.

<table>
<thead>
<tr>
<th>Time (reference Salmonella infection)</th>
<th>D-6</th>
<th>D+2</th>
<th>D+7</th>
<th>D+14</th>
<th>D+21</th>
<th>D+28</th>
<th>D+35</th>
<th>D+42</th>
<th>D+49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella shedding pigs</td>
<td>FB1(-) Salmo(+)</td>
<td>0</td>
<td>8/12</td>
<td>7/8</td>
<td>7/8</td>
<td>7/8</td>
<td>7/8</td>
<td>7/8</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td>FB1(+) Salmo(+)</td>
<td>0</td>
<td>11/12</td>
<td>5/8</td>
<td>4/8</td>
<td>4/8</td>
<td>4/8</td>
<td>4/8</td>
<td>4/8</td>
</tr>
</tbody>
</table>

No effects of FB1 or Salmonella were observed on aerobic flora during the study (Figure 1). The aerobic was comprised between $10^7$ to $10^9$ bacter/gram of faeces without significantly differences.

Figure 1: Numeration of mesophile aerobic flora in faeces of batches FB(-) Salmo(-), FB(+) Salmo(-), FB(+) Salmo(+) and FB(+) Salmo(+) during 49 days after the inoculation.

Bacteriological analyses were completed by fecal flora profile comparison using CE-SSCP. Results were expressed as similarity percentage (Jaccard index) using FB1(-) Salmo(-) as references. The similarity of FB1(-) Salmo(+) batches did not vary significantly during the study (90% of similarities, figure 2). A transient reduction for batch FB1 (+) Salmo(-) was observed: the similarity between the two batches decreased from 95.2% to 80.1% between D-6 to D+22, then reached the control value at D+49. In addition, a transient but marked reduction in the similarity
percentage was observed between control and FB1 (+)-Salmo (+) (Figure 2). Indeed, the similarity of the profiles between these two batches decreases from 89.2% to 74.2% in values, between D-6 to D+2; stabilized between D+2 to D+7 then increased back to high similarity value at D+49.

Figure 2: profile similarities of batches FB(+)-Salmo(-), FB(-)-Salmo(+) and FB(+)-Salmo(+) compared to batch FB(-)-Salmo(-) during 49 days after the inoculation used to Jaccard coefficient and UPGMA average

Discussion
The statistically significant increase in the Sa/So ratio during our study confirmed the fumonisin intoxication of the pigs. This increase confirmed the impact of the 8.5 ppm of FB1 contamination on the sphingolipid metabolism in the kidney, in the liver or in the serum of the pigs after several weeks of exposure. These results agreed with studies showing that the sphingolipid presence increased in the serum from pigs receiving a food containing of low dose of FB1 (higher or equal to 5 ppm) (Riley, et al., 1993).

This study was a first step to approach the influence of a food naturally contaminated by fumonisins on the excretion of Salmonella as it focused on the infectious context (a few days after the inoculation) but also on the finishing pigs. Our experimental conditions showed that a food containing 8.5 ppm FB1 and 2.5 ppm of FB2 did not generate significant effect on the asymptomatic carriage of Salmonella but the observed difference in shedding pig proportion and intensity need to be further investigated in conventional herd condition.

However, a major but also transitory reduction on the similarity of the profiles of the batch exposed to the fumonisins and contaminated by Salmonella was observed compared to the other batches during the acute phase of the Salmonella infection. Therefore, a food containing 8,5 ppm FB1 and 2,5 ppm of FB2 accompanied by an asymptomatic infection with Salmonella would transitory induce deterioration of the digestive balance. This should be add to the immunosupressor effect of FB1 (Bouhet et al., 2006) to take into account the effect of presence of FB in pig feed.

Conclusion
In conclusion, under our experimental conditions, an oral exposure of the pigs to chronic exposure during all the fattening of 8,5 ppm of FB1 in naturally contaminated food induce a transitory deterioration of the digestive flora profiles during the acute phase of the Salmonella infection.

Bibliography