

The effect of fumonisins producing *Fusarium verticillioides* on the microbiota in pig caecum

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Abstract

Fumonisin-producing fungal species, *Fusarium verticillioides*, culture was mixed in the diets of 6 piglets for 9 days (Fumonisin B₁ [FB₁] intake of 17 mg/kg) to investigate whether there is any potential alteration in the caecal bacterial communities between the experimental (with *F. verticillioides*) and control groups (without *F. verticillioides*). Plate count agar culturing technique was applied to measure the amount of aerobic and anaerobic bacteria, *Escherichia coli*, coliforms, *Lactobacillus* spp. and *Clostridium perfringens*. A significant difference was observed between the control and experimental group only in the case of aerobic bacteria on Day 4, 8.60 ± 0.22 compared to 8.06 ± 0.20 ($P < 0.05$), respectively. Quantitative polymerase chain reaction (qPCR) was performed to estimate the DNA copy number of total bacteria, *Bacteroides* and *Prevotella* spp., *Clostridium* spp., *E. coli*, *Enterobacteriales*, *Firmicutes* and *Lactobacillus* spp. Significant differences were observed between the control and experimental group regarding total bacteria on Day 2 and Day 6, *Firmicutes* on Day 2 and *E. coli* and *Enterobacteriales* on Day 4. Regarding the entire feeding time, no significant difference between the two groups was found in all species of investigated bacteria by the culturing technique and qPCR after an 8-day exposure. The present research contributes to the understanding of how microbiota responds to the FB₁ load.

Fumonisin B₁, caecal, culturing, qPCR

Fumonisin is a group of mycotoxins produced by several *Fusarium* species mostly by *Fusarium proliferatum* and *Fusarium verticillioides* (former name *Fusarium moniliforme*) and were first isolated by Gelderblom et al. (1988). Fumonisin B₁ (FB₁) is the most frequently occurring fumonisin, representing about 60% of total fumonisins (Voss et al. 2011). There is a significant structural similarity between the fumonisins and the sphingoid bases sphinganine (Sa) and sphingosine (So). The disruption of sphingolipid metabolism plays an important role in the initiation of a cascade of events which result in the disturbance of various cellular processes, such as the cell membrane function, cell growth, cell differentiation, cell morphology, cell injury and apoptosis; observed both *in vitro* and *in vivo*, all of which contribute to the toxicity and carcinogenicity of FB₁ (Wan et al. 2013). Therefore, fumonisins induce harmful effects on human and animal health (IARC 1993; Marasas 1995; Haschek et al. 2001; Voss et al. 2007).

Pigs are a species highly sensitive to FB₁; the lowest observed adverse effect level (LOAEL) of fumonisin is 200 µg/kg body weight (b.w.) per day (EFSA 2005). Pigs fed fumonisin for at least 93 days developed nodular hyperplasia of the liver and pulmonary vasculature was a target of chronic exposure to fumonisin as reported by Casteel et al. (1994). Fumonisin-treated pigs (20 mg FB₁/kg b.w. daily) had lower cardiac outputs and heart rates than control pigs after 3 days (Constable et al. 2000). Fumonisin B₁ (5 mg FB₁/kg b.w.) had toxicokinetics and toxicological effects on weaned piglets (Dilkin et al. 2010). Cortinovis et al. (2014) demonstrated that FB₁ had inhibitory effects on

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porcine granulosa cell proliferation. European Union's guidance value for both FB₁ and FB₂ is 5 mg/kg per pig (Commission Recommendation 2006/576/EC).

Though many studies on the effect of fumonisins on pigs' health have been conducted, the role of fumonisin bioactivities in the gastrointestinal tract of pigs and especially the influence of fumonisin on intestinal microbiota is not fully understood. A few reports determined the impact of fumonisin on certain gut bacterial species such as *E. coli* and *Salmonella* (Oswald et al. 2003; Devriendt et al. 2010; Burel et al. 2013; Antonissen et al. 2015). In our previous *in vitro* study, alteration of bacterial communities in the pig caecum was not observed (Dang et al. 2017). The aim of this study was to investigate the caecal bacteria in different groups of pigs in *in vivo* conditions.

Materials and Methods

Experimental animals and design

The experimental protocol was authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under permission number XV-1-31/1509-5/2012.

Twelve piglets of the same genotype (9–11 kg per one) were divided into two groups: an experimental group (n = 6) and a control group (n = 6) which would be administered feed with and without *F. verticillioides*, respectively. The temperature and drinking water were set and provided according to the needs of the piglets. A T-cannula was implanted into the caecum, after 7 days of adaptation, following description of Tossenberger et al. (2000), in order to collect the caecal chyme for determination of the effect of FB₁ on the microbiota of the caecum. After 10 days of a regeneration period, a *F. verticillioides* fungal culture was mixed into the ration of the experimental animals, so as to provide a daily FB₁ intake of 17 mg/kg of feed. The duration of the treatment was nine days.

Sampling and processing

During the dietary exposure to *F. verticillioides* (nine days), samples of caecal contents (from 5 to 10 g per sample) were taken on days 0, 2, 4, 6 and 8 through the T-cannula and transferred into sterile tubes which were prepared for microbial culturing. Approximately 1g of sample was subsequently homogenised with 9 ml of peptone salt solution. Then a 10-fold series dilution was conducted from 10⁻¹ to 10⁻⁸. Samples from all tubes were stored in deep freezer (-86 °C) for qPCR confirmation.

Media and enumeration methods

The plate count technique on selected media was applied for determining the number of bacteria. An aliquot (100 µl) from all the diluted series was pipetted and added on the surface of each respectively selected agar to culture bacteria. Five groups of bacteria were enumerated including total aerobic and anaerobic bacteria, coliforms, *E. coli*, *Lactobacillus* spp. and *Clostridium perfringens*. Aerobic and anaerobic bacteria were cultured on commercial blood agar (BA; Bak-Teszt Ltd., Budapest, Hungary). Coliforms and *E. coli* population were estimated on ChromoBio Coliform Agar (BioLab, Budapest, Hungary). The amount of *Lactobacillus* spp. was determined by using de Man Rogosa Sharpe (MRS) agar (BioLab, Budapest, Hungary). For enumeration of *C. perfringens*, the pour plating technique with tryptose sulphite cycloserine (TSC) agar (ISO7937 – VWR Chemical, Budapest, Hungary) was applied. The same amount of diluted sample (100 µl) was pipetted and mixed with TSC agar (10 ml) on Petri dish. Then 10 ml TSC agar were added to cover the thick layer after complete solidification of the previous medium. The temperature and incubation time of culturing were 37 °C and 1 day; while those of *Lactobacillus* spp. culturing were 30 °C and 3 days.

The colony forming units/g (CFU/ g) were calculated using the formula:

$$N = \Sigma C/V \times 1.1 \times d$$

where 'ΣC' is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies; 'V' is the volume of inoculums placed in each dish in millilitres and 'd' is the dilution corresponding to the first dilution retained.

DNA extraction and qPCR

The DNA extraction was carried out with approximately 200 mg of the frozen caecal sample using the QIAamp® DNA Stool Mini Kit (Qiagen, Budapest, Hungary) according to the manufacturer's instructions. The final concentration of the extracts were set to 60 ng/µl.

The standard curve was created by dilution series of purified polymerase chain reaction (PCR) products for *Lactobacillus* spp. and *Firmicutes* whereas the dilution series of plasmid concentration (where plasmids contained a bacteria specific PCR product) was used to prepare the standard curve for total bacteria, *Enterobacteriales*, *E. coli*, *Clostridium* spp. and *Bacteroides* and *Prevotella* spp.

The quantity of bacterial groups was determined by qPCR (Mx3000P, Agilent Technologies, CA, USA) using SYBR Green. The primers for the investigated bacterial groups were selected based on previous literature

(Table 1). The qPCR was conducted in a 25 µl/tube reaction mixture containing 12.5 µl Brilliant II SYBR qPCR Low Rox Master Mix (Agilent Technologies, CA, USA), 0.2 µM of the primer, 10.5 µl sterile diethyl pyrocarbonate (DEPC) treated distilled water, and 1 µl (60 ng/µl) of DNA extract. The qPCR program for total bacteria, *Enterobacteriales*, *E. coli*, *Bacteroides* and *Prevotella* spp. consisted of 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 60 °C whereas the qPCR program for *Firmicutes* was slightly modified as follows, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and finally 1 min at 60 °C. To investigate the amount of *Clostridium* spp., the qPCR program was 3 min at 95 °C, 40 cycles of 40 s at 95 °C, 40 s at 54 °C, 80 s at 72 °C, and the end cycle was 3 min at 72 °C. All samples were measured in triplicates. The bacterial content of the samples was calculated by comparison with the standard curve derived from the dilution series. The obtained copy numbers of the samples were adjusted to 1 g of caecum contents.

Statistical analysis

The IBM SPSS Statistics for Windows software, Version 22.0. (Armonk, NY, IBM Corp., USA) was used for statistical analyses. The comparison of means was performed by independent samples *t*-test and one-way ANOVA with Tukey's *post hoc* test. Repeated measures ANOVA was used to analyse the trend of the amount of bacterial DNA copy number during the different sampling dates.

Table 1. Oligonucleotide sequences used for quantitative polymerase chain reaction in samples from pigs.

Investigated group	Oligonucleotide sequence (5'-3')	Length of amplification product (bp)	References
Total bacteria	Forward: GCAGGCCTAACACATGCAAGTC Reverse: CTGCTGCCTCCCGTAGGAGT	292	Amann et al. (1995); Marchesi et al. (1998); Castillo et al. (2006)
<i>Enterobacteriales</i>	Forward: ATGGCTGTCGTAGCTCGT Reverse: CCTACTTCTTTGCAACCCACTC	177	Sghir et al. (2000); Leser et al. (2002); Castillo et al. (2006)
<i>Escherichia coli</i>	Forward: GGTATGGATCGTTCCGACCT Reverse: GGCAGAATGGTAACACCAGAGT	300	Banu et al. (2010); Pers-Kamczyc et al. (2011)
<i>Bacteroides</i> and <i>Prevotella</i> spp.	Forward: GAAGGTCCCCACATTG Reverse: CAATCGGAGTTCTTCGTG	418	Kim (2011)
<i>Clostridium</i> spp.	Forward: AAAGGAAGATTAATACCGATAA Reverse: ATCTTGGACCGTACTCCCC	722	Mirhosseini et al. (2010)
<i>Lactobacillus</i> spp.	Forward: AGCAGTAGGGAATCTTCCA Reverse: CACCGCTACACATGGAG	340	Walter et al. (2000) Heilig et al. (2002); Su et al. (2008)
<i>Firmicutes</i> spp.	Forward: GGAGYATGTGGTTAATTCGAAGCA Reverse: AGCTGACGACAACCATGCAC	126	Guo et al. (2008)
<i>Delta-</i> and <i>Gamma</i> proteobacteria	Forward: GCTAACGCATTAAGTRYCCCG Reverse: GCCATGCRGCACCTGTCT	189	Yang et al. (2014)

Results

The alteration of the amount of living bacteria in the pigs' caecum is shown in Table 2. Six bacterial types were investigated including aerobe, anaerobe, *E. coli*, coliforms, *Lactobacillus* spp. and *C. perfringens*. Only one difference was observed between the expressed number of aerobic bacteria of control and experimental groups at Day 4, i.e. 8.60 ± 0.22 compared to 8.06 ± 0.20 ($P < 0.05$), respectively, meanwhile no change was found during the trial within each group as well as in trending comparison between two groups. The number of anaerobic bacterial species increased while the amount of *C. perfringens* decreased in a time-dependent manner ($P < 0.05$) within the groups. However, no differences were observed in the entire comparison between the experimental and control groups. No significant changes were observed in the amounts of *E. coli*, coliforms and *Lactobacillus* spp. at all sampling points.

Table 2. Number of bacteria in the caecal chyme of control and fumonisin B₁ exposed pigs measured by culturing (\log_{10} CFU/g, mean \pm SD).

Bacteria	Day 0		Day 4		Day 8	
	C	E	C	E	C	E
Aerobic bacteria	8.44 \pm 0.10	8.06 \pm 0.41	8.60 ^b \pm 0.22	8.06 ^a \pm 0.20	8.56 \pm 0.48	8.13 \pm 0.62
Anaerobic bacteria	8.65 \pm 0.07	8.68 \pm 0.35	9.36 \pm 0.33	9.26 \pm 0.17	9.42 \pm 0.22	9.35 \pm 0.05
<i>E. coli</i>	7.68 \pm 1.12	7.27 \pm 0.21	7.70 \pm 0.29	7.23 \pm 1.08	7.32 \pm 0.47	7.41 \pm 0.95
Coliforms	6.72 \pm 0.96	6.48 \pm 0.64	6.98 \pm 0.44	6.33 \pm 0.09	6.07 \pm 0.56	6.37 \pm 0.55
<i>Lactobacillus</i> spp.	7.86 \pm 0.14	8.16 \pm 0.56	8.44 \pm 0.34	8.17 \pm 0.38	8.35 \pm 0.55	8.16 \pm 0.67
<i>Clostridium perfringens</i>	4.63 \pm 0.06	4.21 \pm 0.62	3.55 \pm 0.68	3.42 \pm 0.91	3.15 \pm 0.61	3.38 \pm 0.89

C - Control group; E - Experimental group; CFU: colony forming unit

^{a,b}: significant ($P < 0.05$) difference between control and experimental groups.

Discussion

Most of the bacterial species in the gastrointestinal tract cannot be identified by classic culturing, only by genetic tools. In the intestine of pig, *Firmicutes* and *Bacteroidetes* are the most dominant phyla (Isaacson and Kim 2012). *Firmicutes* are a major phylum including mostly Gram-positive bacteria such as *Bacilli*, *Clostridia* and *Erysipelotrichia* whereas *Bacteroidetes* consists of many classes of Gram-negative bacteria including *Bacteroides* and *Prevotella* spp. Besides those big phyla, other types of bacteria were investigated by qPCR in this study such as *Enterobacteriales* and *E. coli* (Table 3).

The amount of total bacteria was altered within groups ($P < 0.01$) and significant differences were observed at the sampling points Day 2 and Day 6. Considerable differences between control and experimental groups were presented in *Firmicutes* on Day 2, and *Enterobacteria* and *E. coli* on Day 4. Although the number of scanned bacterial species was changed at different sampling points, when taking into account the whole exposure period and all the investigated bacteria no significant difference could be observed between the control and experimental groups.

It has been reported that FB₁ induces immunosuppression in pigs and that it exerts negative effects on the intestinal epithelial cell viability and proliferation (Bouhet and Oswald 2007; Bracarense et al. 2012) leading to alterations in the gastrointestinal microbial system. Lallès et al. (2009) proved a correlation between FB₁ consumption and the increase of stress protein in the gastrointestinal track in pigs. Cytokine balance was altered after 1 week of dietary exposure to FB₁ (1.5 mg/kg b.w.), interleukin-4 was

Table 3. Number of bacteria in the caecal chyme of control and fumonisin B₁ exposed pigs measured by quantitative polymerase chain reaction (log₁₀ copies number/g, mean ± SD).

Bacteria	Day 0		Day 2		Day 4		Day 6		Day 8	
	C	E	C	E	C	E	C	E	C	E
Total bacteria	12.37 ± 0.18	12.46 ± 0.09	12.48 ^a ± 0.22	12.11 ^a ± 0.27	11.99 ± 0.28	11.95 ± 0.13	12.12 ^a ± 0.28	12.43 ^b ± 0.21	12.48 ± 0.14	12.48 ± 0.08
<i>Bacteroides</i>	9.20 ± 0.32	9.18 ± 0.37	9.28 ± 0.43	8.79 ± 0.49	8.79 ± 0.60	8.62 ± 0.67	8.86 ± 0.67	8.88 ± 0.46	8.79 ± 0.58	8.81 ± 0.71
and <i>Prevotella</i> spp.	8.34 ± 0.58	8.26 ± 0.41	8.31 ± 0.39	8.74 ± 0.50	9.35 ± 0.47	8.98 ± 0.49	9.28 ± 0.32	8.93 ± 0.31	9.19 ± 0.42	9.08 ± 0.31
<i>Clostridium</i> spp.	9.49 ± 0.84	9.64 ± 0.46	9.38 ± 0.51	8.87 ± 0.83	9.65 ^b ± 0.35	8.79 ^a ± 0.50	9.68 ± 0.58	9.14 ± 1.14	9.51 ± 0.59	9.34 ± 0.84
<i>Escherichia coli</i>	10.11 ± 0.86	10.24 ± 0.43	9.98 ± 0.56	9.78 ± 0.74	10.60 ^b ± 0.39	9.88 ^a ± 0.38	10.52 ± 0.55	9.82 ± 1.05	9.71 ± 0.52	9.61 ± 0.84
Enterobacteria	10.55 ± 0.14	10.52 ± 0.07	10.52 ^b ± 0.14	10.36 ^a ± 0.10	10.36 ± 0.11	10.30 ± 0.08	10.26 ± 0.17	10.27 ± 0.15	10.24 ± 0.09	10.26 ± 0.05
<i>Firmicutes</i> spp.	10.34 ± 1.07	10.06 ± 0.91	10.12 ± 0.87	10.39 ± 0.47	9.88 ± 0.87	10.19 ± 0.57	9.98 ± 0.75	10.16 ± 0.50	10.07 ± 0.98	9.91 ± 0.79
<i>Lactobacillus</i> spp.										

C - Control group; E - Experimental group

^{a, b} Significant ($P < 0.05$) difference between control and experimental groups

decreased, interferon-gamma synthesis was increased (Taranu et al. 2005). Bouhet et al. (2006) reported that FB₁ (0.5 mg/kg b.w. for 7 days) effected the intestinal immune response by reducing the level of interleukin IL-8. However, the effects of FB₁ on bacterial species are controversial based on the few studies in the literature. Becker et al. (1997) treated certain bacterial strains including *E. coli* and *Salmonella* with FB₁ but did not observe any inhibition of the bacterial growth while FB₁ (0.5 to 1 mg/kg body weight) could predispose in the colonization of pathogenic *E. coli* in pigs (Oswald et al. 2003; Devriendt et al. 2010). A significant change in the faecal microbiota composition was observed in pigs co-infected with *Salmonella* and fumonisins (11.8 mg/kg FB₁ + FB₂ of feed) for 9 weeks (Burel et al. 2013). In this study, the growth of bacteria including *E. coli* in control groups was similar to experimental groups though there were differences in some points of sampling.

Microbial communities can be distinguished by the factors related to breed, season or sampling time (Pajarillo et al. 2014). The amount of bacteria in the intestine can also be affected by different diets (Frese et al. 2015). The stability of the amount of caecal bacteria in this study showed that the gut microflora might have adapted to the environmental change (i.e. toxin exposure). Fumonisin producing *F. verticillioides* can modify the bacterial growth but only within a short period while no change of investigated bacteria was observed on the 8th day of the fungal treatment. A longer time of exposure should be performed in order to achieve more information on the influence of FB₁ on the intestinal microorganisms.

In practice, *F. verticillioides* frequently infests cereals. Since its product FB₁ affects a variety of cell signalling molecules manifesting through e.g. carcinogenesis or apoptosis and is linked to toxicosis in domestic animals, it is important to explore how microbiota responds to a FB₁ load. The accumulating knowledge can lead to the possibility of educated intervention via bacterial community to avoid the effects of FB₁.

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