ORIGINAL PAPER

In vitro Interaction between Fumonisin B₁
and the Intestinal Microflora of Pigs

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A b s t r a c t

The caecal chyme of pigs was incubated anaerobically in McDougall buffer with and without fumonisin B₁ (5 µg/ml) for 0, 24 and 48 h. The plate count agar technique was applied for enumerating the amount of bacteria including aerobic, anaerobic bacteria, coliform, *Escherichia coli* and *Lactobacillus* sp. The quantitative polymerase chain reaction was also performed to estimate the number of copies of the total bacteria, *Lactobacillus*, *Bacteroides* and *Prevotella*. No significant differences in the amount of bacterial groups between the experimental (buffer, chyme, and fumonisin B₁) and control 1 groups (buffer + chyme) were observed in both methods. Fumonisin B₁ and hydrolysed fumonisin B₁ concentration were analysed by liquid chromatograghy – mass spectrometry. There was no significant difference in FB₁ concentration between the experimental and the control 2 group (buffer and fumonisin B₁) at 0 h incubation, 5.185 ± 0.174 µg/ml compared with 6.433 ± 0.076 µg/ml. Fumonisin B₁ concentration in the experimental group was reduced to 4.080 ± 0.065 µg/ml at 24 h and to 2.747 ± 0.548 µg/ml at 48 h incubation and was significantly less than that of in the control group. Hydrolysed fumonisin B₁ was detected after 24 h incubation (0.012 ± 0 µg/ml). At 48 h incubation time, hydrolysed fumonisin B₁ concentration was doubled to 0.024 ± 0.004 µg/ml. These results indicate that fumonisin B₁ can be metabolised by caecal microbiota in pigs though the number of studied bacteria did not change.

Introduction

The fumonisins are known as important mycotoxins produced mostly by several *Fusarium* species which are found mainly in maize and its products all over the world. Because of the similarity between fumonisins and the sphingoid bases sphinganine (Sa) and sphingosine (So), fumonisin disrupts the sphingolipid metabolism and impacts on animal health. Pigs are high sensitive species to fumonisin B₁ (FB₁), the lowest observed adverse effect level (LOAEL) of fumonisin is 200 µg/kg b.w. per day (EFSA, 2005). It is very important to know the activities of fumonisin from the digestion on the cell systems. However, only a few studies have been conducted to determine fumonisin activities in the gastrointestinal tract of pigs, especially interaction between fumonisin and gut microbiota. Only one report showed the fumonisin was metabolised by the caecal microorganism (Fodor *et al.*, 2007) while there was no complete research about the effect of fumonisins on the intestinal microbiota in pigs. Some *in vivo*,

not *in vitro* studies were carried out to estimate the impact of fumonisin on individual microbial species such as *Escherichia coli* and *Salmonella* sp. According to the results, intestinal colonization of pathogenic *E. coli* strain was significantly increased when pigs were treated with 0.5 mg of FB₁/kg of body weight for 7 days (Oswald *et al.*, 2003) while no change of *Salmonella enterica* growth was presented in pig intestine with the moderate dose of fumonisin (8.6 mg FB₁ and 3.2 mg FB₂ per kilogram feeding intake), it transiently affected the digestive microbiota balance (Burel *et al.*, 2013). It is essential to have more information about the impact of fumonisin mycotoxin on gastrointestinal microbiota in pigs. The gut microbiota balance should be concerned as a crucial factor keeping body health. From the gut microbiota ecosystem point of view, it is good to understand the activities of all kinds of bacteria rather than one or few particular types of them when treated with fumonisins. In the presented *in vitro* study, we investigated the interaction between FB₁ and caecal microorganism in pigs i.) to seek signs

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of biological effect on FB$_1$ induced by gut microbiota and ii.) to determine the impact of FB$_1$ on the growth of pig caecal bacteria.

Experimental

Materials and Methods

Sampling and processing. Samples of caecal content were collected from adult pigs (n = 2; Hungarian large white race) right after slaughtering from a slaughter house and transferred into sterile bottles. The bottles were put in anaerobic plastic bags with Anaerocult gas generator (Merck, Darmstadt, Germany). The pre-incubated (24 h/37°C/anaerobic) McDougall buffer solution (9.8 g NaHCO$_3$, 3.7 g anhydrous Na$_2$HPO$_4$, 0.57 g KCl, 0.47 g NaCl, 0.12 g MgSO$_4$·7H$_2$O, 0.04 g CaCl$_2$ and 1000 ml aquadest; pH 8.3) were prepared to homogenise samples and make the solution for control groups.

The experiment was designed to 3 groups as shown in Table I. Two control groups were set up. The first controls contained buffer and chyme, while the second controls were prepared including buffer and FB$_1$.

Caecal chyme was homogenised and divided into the first control and experimental groups. An aliquot of 3.33 g of caecal chyme was suspended in pre-incubated McDougal buffer tubes (experimental and the first control group). After a pre-incubation for four hours at 37°C, FB$_1$ (50 µg/g; Sigma-Aldrich, Darmstadt, Germany) was added to each tube (experimental and the second control groups) to get a final concentration of 5 µg/ml. Samples were taken at 0, 24 and 48 h of anaerobic incubation for determination of bacterial numbers and FB$_1$ concentration.

Media and enumeration methods. The plate count technique on selected media was applied for determining the amount of bacteria. Approximately 1 g of post-incubated sample was collected and subsequently homogenised with 9 ml of peptone salt solution. Then the 10-folds series dilution was conducted from 10^{-1} to 10^{-8}. An aliquot (100 µl) was pipetted and add on the surface of each respective selected agar to culture bacteria. Five groups of bacteria were enumerated including aerobic bacteria, anaerobic bacteria, coliform, *E. coli* and *Lactobacillus* sp. The aerobic and anaerobic bacteria were cultured in commercial blood agar (BA; Bak-Teszt Ltd., Budapest, Hungary). Coliform and *E. coli* population were estimated on ChromoBio Coliform Agar (BioLab). The amount of *Lactobacillus* sp. was determined by using MRS agar (BioLab).

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

$$N = \Sigma C/Vx1, 1xd$$

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

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 according to the manufacturer's instructions. DNA concentrations were measured using Smart Spec Plus Spectrophotometer (Maestrogen Inc.) based on the ratio of absorbance at 260 nm and 280 nm.

The standard curve was created by dilution series of purified PCR products for *Lactobacillus* sp. while the dilution series of plasmid concentration were used to prepare the standard curve for total bacteria, *Bacteroides* and *Prevotella*.

The quantity of total bacteria, *Lactobacillus* sp., *Bacteroides* and *Prevotella*, were determined by Quantitative PCR (QPCR) using SYBR Green. The primers for investigated bacterial groups were selected based on previous literature (Table II). QPCR was conducted in a 25 µl/tube reaction mixture containing 12.5 µl Brillant II SYBR QPCR Low Rox Master Mix (Agilent Technologies, CA, USA), 0.2 µM of each primer, 10.5 µl sterile DEPC treated distilled water and 1 µl of DNA extract. The PCR program consisted of 10 min at 95°C, 40 cycles of 30 sec at 95°C, 1 min at 60°C. All samples were measured in triplicates. The bacterial content of the sam-

Table I
Experimental design.

Incubation (h)/treatment	Experimental group (Buffer+Chyme+FB$_1$)	Control 1 group (Buffer+Chyme)	Control 2 group (Buffer+FB$_1$)
0 h	4	4	4
24 h	4	4	4
48 h	4	4	4
Description	12 × 3,33 g chyme 12 × 5,67 ml buffer 12 × 1 ml 50 µg/g FB$_1$	12 × 3,33 g chyme 12 × 5,67 ml buffer 1 ml H$_2$O	12 × 9 ml buffer 12 × 1 ml 50 µg/g FB$_1$

Table II
Oligonucleotide sequences used for QPCR in caecal samples from pigs.

Investigated group	Oligonucleotide sequence (5'-3')	Amplicon length (bp)	Refs.
Total bacteria	Fwd: GCAGGCCTAACACATGCAAGTC Rev: CTGCTGCCTCCCGTAGGAGT	292	Castillo *et al.*, 2006; Marchesi *et al.*, 1998 Amann *et al.*, 1995
Bacteroides and *Prevotella*	Fwd: GAAGGTCCCCCACATTG Rev: CAATCGGAGTTCTTCGTG	418	Kim, 2011 Bartosch *et al.*, 2004
Lactobacillus sp.	Fwd: AGCAGTAGGGAATCTTCCA Rev: CACCGCTACACATGGAG	340	Su *et al.*, 2008; Walter *et al.*, 2000 Heilig *et al.*, 2002

ples was calculated by comparison with the standard curve derived from dilution series. The obtained copy numbers of the samples were adjusted to one gram of caecum contents.

Mycotoxin extraction and analysis. For FB$_1$ extraction, the post-incubated samples from the experimental group and the control 2 group (Table I) were diluted 2-fold (7 ml sample and 7 ml distilled water) and centrifuged for 5 minutes (3000 rpm). The supernatant was used for FB$_1$ extraction followed by the modified protocol of Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA) (Szabó-Fodor *et al.*, 2014). The preconditioning column was conducted with 2 ml of methanol then 2 ml of distilled water. The diluted chyme (2 ml) was subsequently loaded onto the columns then washed again with 2 ml of distilled water. The elution of FB$_1$ was completed by 2 ml of water/acetonitrile mixture, 1:1 v/v.

Liquid chromatography and mass spectrometry (LC-MS) analysis were performed by a Shimadzu Prominence UFLC separation system equipped with an LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) with the electrospray source. Optimised mass spectra were obtained with an interface voltage of 4.5 kV, a detector voltage of 1.05 kV in negative mode, 1.25 kV in positive mode. Samples were analysed on a Phenomenex Kinetex 2.6 μ X-C18 column (100 mm × 2.1 mm). The column temperature was set to 50°C; the flow rate was 0.3 ml/minute. The gradient elution was performed using LC-MS grade water (VWR Hungary, Debrecen) (eluent A) and methanol (eluent B), both acidified with 0.1% acetic acid. One μl of each samples were analysed with a gradient: (0 min) 5% B, (3 min) 60% B, (8 min) 100% B, followed by a holding time of 3 min at 100% eluent B and 3 min column re-equilibration at eluent A. Romer Mix 3 (containing FB$_1$ + FB$_2$ at 50 mg/l) and HFB$_1$ primary stock solution used as reference. MS parameters: source block temperature 90°C; desolvation temperature 250°C; heat block temperature 200°C; drying gas flow 15.0 l/min. Detection was performed using selected ion monitoring (SIM) mode.

The efficiency of FB$_1$ conversion to fully hydrolysed FB$_1$ (HFB$_1$) was calculated on the basis of the molecular weight of the compounds (FB$_1$: 721 g/mol; HFB$_1$: 405 g/mol) and described as below:

$$\frac{\text{Hydrolysed fumonisin B1 (mol/g)} \times 721\,\text{g/mol}}{405\,\text{g/mol} \times \text{Fumonisin B1 (mol/g)}}$$

Statistical analysis. The R i386 3.1.2 program was applied for statistical analyses. The comparative means were performed by Independent Samples t-Test, one-way ANOVA with Tukey post-hoc test and non-parametric Kruskal-Wallis test if the normal distribution was not presented.

Results and Discussion

Effect of microflora on fumonisin B$_1$. At 0 h incubation time, no significant FB$_1$ concentration difference between the experimental group (buffer, caecal content, FB$_1$) and control 2 group (buffer, FB$_1$) was observed; 5.185 ± 0.175 μg/ml compared with 6.433 ± 0.076 μg/ml, respectively. FB$_1$ concentration in experimental groups was significant lower than control-2 group after 24 h and 48 h incubation period, 4.080 ± 0.065 μg/ml and 2.747 ± 0.548 μg/ml compared to 6,338 ± 0,108 μg/ml and 4.587 ± 0.085 μg/ml, respectively. FB$_1$ concentration also decreased during incubation time in the experimental group (Fig. 1). HFB$_1$ concentration has
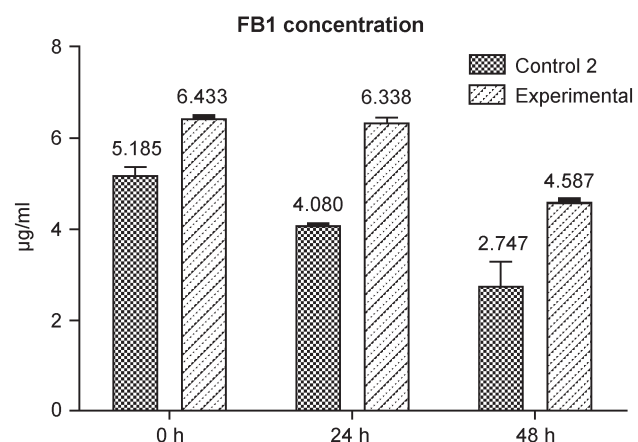

Fig. 1. Fumonisin B$_1$ concentration in experimental groups and control 2 groups during the incubation time.
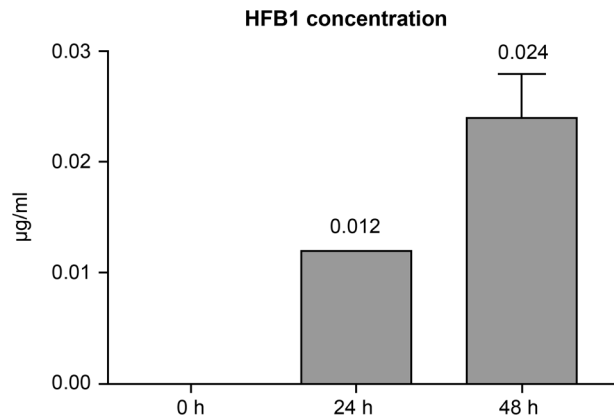
HFB1 concentration

Fig. 2. Hydrolysed fumonisin B_1 concentration in experimental groups during the incubation time.

also been determined at different incubation times. Due to the appearance of main products of the metabolism (HFB_1) only in the experimental group (Fig. 2), we can conclude that FB_1 may be metabolised by microbiota in the caecum of the pig.

The capability of bacteria to influence fumonisins was by Niderkorn *et al.* (2009) and Zoghi *et al.*, (2014). Peptidoglycan, the component of bacterial cell wall, plays a crucial role in binding many mycotoxins including fumonisins. *Lactobacillus* sp. is the class of bacteria having a significant impact on fumonisins. The FB_1 level in maize was decreased by lactic acid bacterial activity after 3-day fermentation (Mokoena *et al.*, 2005). To determine the effect of the microorganism to fumonisins, most of the studies were conducted to estimate the impact of bacteria to fumonisin produced by *Fusarium* sp. such as binding or inhibition of fumonisin production while few of them were concerned with fumonisin metabolism. The concentration of FB_1 was reduced by *Lactobacillus paracasie* subsp. Paracasie after 20-day incubation (70.5 μl/ml compared with 300 μl/ml FB_1 in the control group) and *L. paracasie* subsp. Paracasie could inhibit FB_1 production in

a 10-day incubation period (Gomah and Zohri, 2014). Becker *et al.* (1997) reported that FB_1 was not degraded by *Enterococcus faecium* while the binding of FB_1 and FB_2, up to 24 and 62%, respectively by *Enterococcus* sp. was determined (Niderkorn *et al.*, 2007).

In agreement with former results reported by Fodor *et al.* (2007), the conversion of FB_1 to HFB_1 was less than 1% where there was no change in the degree of the conversion of FB_1 to aminopentol (fully hydrolysed FB_1). In this study, conversion of FB_1 to HFB_1 increased significantly from 0.33% to 0.66% after 24 h and 48 h incubation time, respectively. Differences in the HFB_1 related results can be explained on the basis of the different bacterial ecosystem in the gut of experimental pigs. The various structures of gut microbiota may be derived from different diets, time of the sampling or individual enterotypes of the porcine gut microbiota (Pajarillo *et al.*, 2014; Frese *et al.*, 2015).

Effect of fumonisin B_1 on caecal microbiota in pigs. Five groups of bacteria were quantitatively determined by microbial culturing including aerobic bacteria, anaerobic bacteria, coliform, *E. coli* and *Lactobacillus* sp. There was no significant difference in the groups without FB_1 during the period of the incubation time except the anaerobic bacteria group. The $\log_{10}$ number of anaerobic bacteria decreased from 9.046 ± 0.036 (0 h incubation) to 8.389 ± 0.143 (48 h incubation) (Table III). In the caecal bacteria with FB_1 groups, reduction of the $\log_{10}$ number of anaerobic bacteria were identified, from 9.017 ± 0.054 to 8.340 ± 0.082, while there was an increase in *Lactobacillus* sp. group from 7.764 ± 0.040 to 8.006 ± 0.106 after 48 h incubation (Table III). Nonetheless, there was no detectable change in microbial culturing method between the groups of caecal bacteria with and without FB_1 during the incubation time.

The quantitative PCR was also performed to determine the effect of FB_1 on total bacteria, *Bacteroides* and *Prevotella* and *Lactobacillus* sp. The $\log_{10}$ copy-numbers were applied for data analysis (Table IV). The $\log_{10}$ of

Table III

Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B_1 measured by culturing ($\log_{10}$ CFU[1]/g, means ±SD).

Bacteria	Period of the incubation time					
	0 hour		24 hour		48 hour	
	Experimental group	Control 1	Experimental group	Control 1	Experimental group	Control 1
Aerobic	7.49 ± 0.09	7.58 ± 0.07	7.55 ± 0.15	7.49 ± 0.258	7.26 ± 0.22	7.31 ± 0.19
Anaerobic	9.02 ± 0.05^c	9.05 ± 0.04^c	8.74 ± 0.19^b	8.76 ± 0.05^b	8.34 ± 0.08^a	8.39 ± 0.14^a
E. coli	5.89 ± 0.07	5.87 ± 0.07	5.58 ± 0.11	5.99 ± 0.33	6.16 ± 0.83	5.87 ± 0.66
Coliforms	5.33 ± 0.06	5.39 ± 0.12	5.44 ± 0.11	5.69 ± 0.29	5.99 ± 0.86	5.84 ± 0.55
Lactobacillus	7.76 ± 0.04^a	7.87 ± 0.09	7.99 ± 0.06^b	8.04 ± 0.09	8.01 ± 0.11^b	7.93 ± 0.12

[1]CFU: colony forming unit
[a, b, c] significant (P < 0.01) difference between incubation times within groups.

Table IV
Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B$_1$
measured by QPCR (log$_{10}$ copies number/g, means ±SD).

Bacteria	Period of the incubation time					
	0 hour		24 hour		48 hour	
	Experimental group	Control 1	Experimental group	Control 1	Experimental group	Control 1
Total bacteria	11.52 ± 0.16[a]	11.33 ± 0.38	11.91 ± 0.03[b]	11.68 ± 0.21	11.79 ± 0.05[b]	11.66 ± 0.13
Bacteroides and *Prevotella*	7.41 ± 0.14[a]	7.32 ± 0.28[a]	7.83 ± 0.13[b]	7.95 ± 0.16[b]	7.97 ± 0.11[b]	7.83 ± 0.12[b]
Lactobacillus	9.80 ± 0.25[a]	9.61 ± 0.40[a]	11.23 ± 0.17[b]	11.35 ± 0.11[b]	11.33 ± 0.14[b]	11.13 ± 0.15[b]

[a, b] significant (P < 0.05) difference between incubation times within groups.

Lactobacillus, *Bacteroides* and *Prevotella* in control 1 and experimental groups augmented after 24 h incubation (P < 0.05). A number of total bacteria was stable during the incubation time in the control groups while there was an increase in the experimental group from 11.520 at 0 h to 11.912 at the 24 h incubation. However, no significant difference between the control groups and the experimental groups in all kinds of investigated bacteria was observed. FB$_1$ did not affect the number of caecal bacteria in pigs.

As we have detected both in the microbial culture and in QPCR experiment during the incubation time, the anaerobic bacteria decreased while the amount of *Lactobacillus* sp. increased. According to QPCR results the amount of *Bacteroides* and *Prevotella* also increased. The primary difference between the results of two methods is that anaerobic bacteria enumerating by culture is alive organisms whereas *Lactobacillus* sp., *Bacteroides* and *Prevotella* estimating by QPCR based on DNA copy-number. The decline of other, not investigated anaerobic bacterial species (*i.e. Clostridium* sp.), might be another reason in this situation. Further experiments should be focused on other kinds of anaerobic bacteria or all bacterial species using a next generation sequencing approach.

To the best of our knowledge, there was is no complete report about the effect of fumonisin on caecal bacteria in pigs. Becker *et al.* (1997) isolated some strains of *Lactobacillus* sp. from pig intestine and determined the effect of FB$_1$ (50 and 500 μM) on the growth of these strain by turbidometric Bioscreen system. As shown in the report, no difference in the growth kinetics between the experimental and control groups was observed. The DNA of *E. coli* was not affected by FB$_1$ (Knasmüller *et al.*, 1997) and the number of *E. coli* showed no change in the presence of FB$_1$ in this study. However, the intestinal colonisation by pathogenic *E. coli* in pigs treated FB$_1$ was strengthened in an *in vivo* experiment (Oswald *et al.*, 2003). The indirect impact of fumonisin on bacteria was also demonstrated in some documents; because of immune suppressive effects and

decrease the specific antibody response of pathogenic microorganisms (Taranu *et al.*, 2005; Iheshiulor *et al.*, 2011), fumonisin can influence activities of colonised bacteria in the body such as *E. coli* and *Salmonella* sp. (Deshmukh *et al.*, 2005; Burel *et al.*, 2013).

Fumonisin B$_1$ was metabolised by pig caecal microorganisms. The reduction of FB$_1$ concentration in chyme containing groups was sharper than it was in control 2 group. FB$_1$ concentration decreased while the HFB$_1$ increased. The amount of screened bacterial species has not changed between the groups with and without FB$_1$. During the period of incubation, the total number of cultured anaerobic bacteria decline while *Lactobacillus* sp. increased. Anaerobic bacteria such as *Lactobacillus* sp., *Bacteroides* and *Prevotella* had tended to increase revealed by QPCR. FB$_1$ did not impact on the growth of investigated bacteria. Other kinds of microorganisms should be concerned in the similar experiments and the interaction between fumonisins and gut microbiota in the *in vivo* experiments is to be conducted.

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