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HUU ANH DANG

UNIVERSITY OF KAPOSVÁR

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Department of Physiology and Animal Hygiene

The head of the Doctoral School

Prof. Dr. KOVÁCS MELINDA

Corresponding member of the Hungarian Academy of Sciences

Supervisor

Dr. ZSOLNAI ATTILA

**INTERACTION OF FUMONISIN MYCOTOXINS AND
GASTROINTESTINAL MICROBIOTA IN
SHEEP AND SWINE**

Created by

HUU ANH DANG

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ABBREVIATION

BA:	Blood agar
bw:	body weight
CFU:	Colony forming unit
CPC:	Centrifugal partition chromatography
DON:	Deoxynivalenol
DNA:	Deoxyribonucleic acid
ELEM:	Equine leukoencephalomalacia
ELISA:	Enzyme-linked immunosorbent assay
FA, FB, FC, FP:	Fumonisin A, B, C, P
GC-MS:	Gas chromatography-mass spectrometry
GIT:	Gastrointestinal tract
HPLC:	High performance liquid chromatography
IAC:	Immunoaffinity columns
LC-MS:	Liquid chromatography-mass spectrometry
Mab:	Monoclonal antibodies
MRS:	de Man, Rogosa, Sharpe
NGS:	Next generation sequencing
PCR:	Polymerase chain reaction
PPE:	Porcine pulmonary edema

qPCR:	Quantitative polymerase chain reaction
RNA:	Ribonucleic acid
SPE:	Solid phase extraction
SAX:	Strong anion-exchange
TLC:	Thin layer chromatography
TSC:	Tryptose sulfite cycloserine
ZEA:	Zearalenone

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2. Introduction

The fumonisins, first isolated by Gelderblom et al. (1988), are a group of mycotoxins produced by many *Fusarium* species mostly by *Fusarium proliferate* and *Fusarium verticillioides* (former name is *Fusarium moniliforme*). It was believed that fumonisins were only produced by *Fusarium* species until the year 2000. However, other fungi can also synthesize fumonisins such as *Aspergillus niger* (Frisvad et al. 2007) and *Aspergillus awamori* (Varga et al. 2010). Fumonisins are found mainly in maize, all over the world. The presence of fumonisin B₁ (FB₁) is the most frequent among fumonisins in maize, representing about 60% of total fumonisins (Voss et al., 2011).

In Europe, FB₁ concentrations varied from 0.007 to 250 mg/kg in maize, and 0.008 to 16 mg/kg in maize products (Scientific Committee on Food, EU Commission, 2000). The evaluation of fumonisin exposure in human has been reported in some countries - for example, the mean dietary consumption in Switzerland was 0.03 µg/kg bw/day. In the Netherlands it was between 0.006 and 7.1 µg/kg bw/day (EHC, 2000). The prevalence of FB₁ in maize and cornflake samples in Europe was 66% and 46%, respectively (SCOOP, 2003). During an 8-year period (2004-2012), more than 17000 samples of feed and feed raw materials from all over the world were analyzed for contamination with aflatoxins, ochratoxin A, zearalenone, deoxynivalenol and fumonisins. As a result, in Central Europe, the prevalence of fumonisins was 51% (number of samples analysed for FBs: 206) whereas in Southern Europe it was 70% (total number of samples: 233) and no data were available for Northern Europe (Schatzmayer and Streit, 2013). The EU regulations for mycotoxin contamination in human food are among the strictest over the

world. The European Commission set maximum limits for the sum of FB₁ and FB₂, namely 1000 µg/kg for maize and maize-based foods intended for direct human consumption, 800 µg/kg for maize-based breakfast cereals and maize-based snacks, 200 µg/kg for processed maize-based foods and baby foods for infants and young children (Commission Regulation (EC) No 1881/2006). For animals, the European Union's guidance values are 20 mg/kg for FB₁+ FB₂ in compound feed for poultry; 5 mg/kg for pig, horse, rabbits and pet animal; 50 (20) mg/kg for ruminants (young) (Commission Recommendation, 2006/576/EC).

The chemical structure of fumonisins consists of a stable carbon chain which is similar to sphinganine (Sa) and sphingosine (So). Therefore, fumonisins can interrupt the sphingolipid synthesised process playing a crucial role in lipoprotein, cell wall synthesis and metabolism regulation. In relatively high doses and after a prolonged feeding of fumonisins, have led to harmful effects on human and animal health (Quinn et al., 2011) such as cause esophageal cancer in human (IARC, 1993), porcine pulmonary edema (PPE), equine leukoencephalomalacia (ELEM) and liver damage in multiple species including pigs, horses, cattle, rabbits, and primates; kidney damage in rats, rabbits, and sheep (Smith, 2007). While pigs are the most sensitive group of animals to fumonisin, the ruminants are the most tolerant species of this toxin. Pigs fed fumonisin for at least 93 days developed nodular hyperplasia of the liver and pulmonary vasculature is a target of chronic exposure to fumonisin as reported by Casteel et al. (1994). Fumonisin treated pigs (20 mg FB₁/kg bw daily) had lower cardiac outputs and heart rates than control pigs after 3 days (Constable et al. 2000). Cortinovis et al. (2014) demonstrated that FB₁ had inhibitory effects on porcine granulosa cell proliferation. Regarding the slight effect of fumonisins on ruminant species, some studies were carried out

to see how fumonisins induce a negative effect on ruminants. In cattle, FB₁ causes liver damage (Osweiler et al., 1993), hepatotoxic and nephrotoxic in milk-fed calves with 1 mg/kg intravenously (Mathur et al., 2001). The decrease in feed intake and milk production as well as the increase of serum aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) were observed in cow treated with FB₁ (Richard et al., 1996; Diaz et al., 2000; Baker and Rottinghaus, 1999). FB₁ was proven in cytotoxic capability via an effect on the oxidative status of bovine peripheral blood mononuclear cells (Bernabucci et al., 2011). In sheep, FB₁ induce liver damage and renal toxicity (Edrington et al, 1995). FB₁ can cause immunosuppression and immunostimulation in mice immunized with sheep red blood cells (Martinova and Merrill, 1995). It is hypothesized that several biochemical processes have occurred for prohibiting FB₁ production, binding, metabolism or degradation FB₁ in the rumen. Then less FB₁ can pass through the four-chambered stomach to arrive at the small intestine where it can be absorbed mostly and cause toxic effects.

Many experiments were performed to understand the impact of fumonisin on animal health while only a few studies (most of them used *in vitro* methods) about fumonisins and the gut microbiota were conducted (Becker et al., 1997; Fodor et al., 2007; Loiseau et al., 2007; Burel et al., 2013). Gastrointestinal tract (GIT) is the first target organ of these toxic compounds entering the body via feed/food and fully understanding of the fumonisin activities in the GIT is necessary to explore the pathway of biotransformation of fumonisin in the body. This research will be carried out to estimate the interaction of fumonisin mycotoxins and the gastrointestinal microbiota in sheep and swine using *in vitro* and *in vivo* experiments.

3. Literature review

3.1. Fumonisin mycotoxins and the analytic methods

3.1.1. Chemical structure of fumonisins

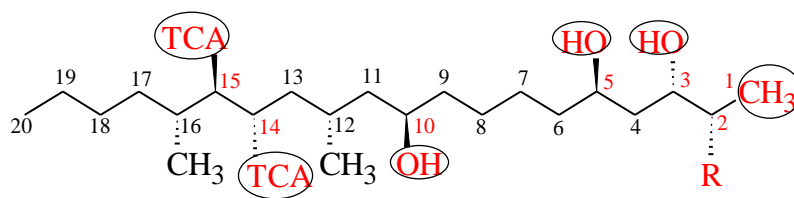
Four groups of fumonisins (FA, FB, FC and FP) were classified based on the structure of backbone and that of the functional groups at positions C1, 2, 3 and 10 (Musser and Plattner, 1997). Fumonisin B group is the most abundant among fumonisins produced by fungal species (Table 1). Theoretically, there are thousands of isomers of fumonisins those can be synthesized based on chiral centres of fumonisin structure (Bartók et al., 2010). More than 100 isomers and stereoisomers of fumonisins were asserted by researchers (Rheeder et al., 2002; Bartók et al., 2008; Varga et al., 2010; Bartók et al., 2014). The chemical structure of fumonisins consists of a 19-carbon amino-polyhydroxy alkyl chain (fumonisin C) or a 20-carbon amino-polyhydroxyalkyl chain (fumonisin A, B, P) and some different chemical groups (*N*-acetyl amide, amine, tricarboxylic) depending on the type of fumonisin analogue (Table 2, Fig. 1). Basically, compounds at the carbon position number 14 and 15 are *tricarballic acid* (TCA) and they can be found in all groups of fumonisins except some isomers. Different fumonisin analogues are also distinguished by interchange hydrogen and hydroxide in C-3 and C-10 positions. The highest extent of differences among the chemical structures of fumonisins is in C-2 position. These groups are the *N*-acetyl amide (NHCOCH₃) in fumonisin A group, the amine (NH₂) in fumonisin B and C, and the 3-hydroxypyridinium (3HP) moiety in fumonisin P.

**Table 1. Fumonisin producing fungal species
(adapted from Rheeder et al., 2002)**

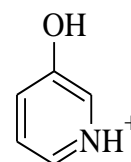
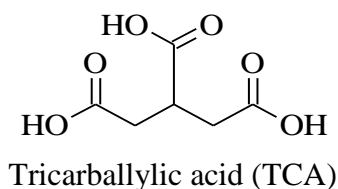
Fungal species	FAs	FBs	FCs	FPs	Sources
<i>Aspergillus niger</i>		X			Frisvad et al. 2007
<i>Aspergillus awamori</i>		X			Varga et al. 2010
<i>Fusarium anthophilum</i>		X			Nelson et al. 1992
<i>Fusarium dlamini</i>		X			Nelson et al. 1992
<i>Fusarium fujikuroi</i>		X			Desjardins et al. 2000
<i>Fusarium globosum</i>		X			Sydenham et al. 1997
<i>Fusarium napiforme</i>		X			Nelson et al. 1992
<i>Fusarium nygamai</i>	X	X	X	X	Musser and Plattner 1997
<i>Fusarium oxysporum</i>		X	X		Abbas et al. 1995 Seo et al. 1996
<i>Fusarium polyphialidicum</i>		X			Abbas et al. 1995
<i>Fusarium proliferatum</i>	X	X	X	X	Castella et al. 1999 Musser and Plattner 1997
<i>Fusarium sacchari</i>		X			Leslie et al. 1992
<i>Fusarium subglutinans</i>		X			Leslie et al. 1992
<i>Fusarium thapsinum</i>		X			Klittich et al. 1997
<i>Fusarium verticillioides</i>	X	X	X	X	Alberts et al. 1990 Branham et al. 1993 Musser and Plattner 1997

**Table 2. Functional groups of the fumonisin analogues
(adapted from Musser and Plattner, 1997)**

Fumonisin	Carbon position				Formula
	C1	C2	C3	C10	
FA ₁	CH ₃	NHCOCH ₃	OH	OH	C ₃₆ H ₆₁ NO ₁₆
FA ₂	CH ₃	NHCOCH ₃	OH	H	C ₃₆ H ₆₁ NO ₁₅
FA ₃	CH ₃	NHCOCH ₃	H	OH	C ₃₆ H ₆₁ NO ₁₅
FB ₁	CH ₃	NH ₂	OH	OH	C ₃₄ H ₅₉ NO ₁₅
FB ₂	CH ₃	NH ₂	OH	H	C ₃₄ H ₅₉ NO ₁₄
FB ₃	CH ₃	NH ₂	H	OH	C ₃₄ H ₅₉ NO ₁₄
FC ₁	H	NH ₂	OH	OH	C ₃₃ H ₅₇ NO ₁₅
FP ₁	CH ₃	3HP	OH	OH	C ₃₉ H ₆₂ NO ₁₆ ⁺
FP ₂	CH ₃	3HP	OH	H	C ₃₉ H ₆₂ NO ₁₅ ⁺
FP ₃	CH ₃	3HP	H	OH	C ₃₉ H ₆₂ NO ₁₅ ⁺



Fumonisin



3-Hydroxypyridinium (3HP)

Figure 1: Chemical structure of fumonisins

3.1.2. The metabolized fumonisin products and their toxicity

The chemical structure of the carbon backbone of fumonisins is quite stable. However, the functional groups which connect to fumonisin backbone can be affected by chemical or physical factors or by an enzyme (Humpf and Voss, 2004; Heiln et al., 2010; Formenti et al., 2012). In fact, changes of those compounds among fumonisin analogues are the solid evidence for the impact of the structure of fumonisins. Up to now, most of the chemical structure studies focus on FBs, especially FB₁. The changeable functional groups are in the position of C2, C5, C10 and TCAs at C14 and C15.

Because of the availability of the amine group in FB₁, the Maillard reaction between an amino acid and a reducing sugar with the addition of heat was considered as the method to affect the chemical structure of FB₁. It was believed that the toxicity of FB₁ derived from the amine group because the N-acetyl-FB₁ is non-toxic (Gelderblom et al., 1993). Murphy et al. (1996)

suggested using sugar such as fructose or glucose to block the amine group and detoxify FB₁. Two derivatives can be converted and isolated from FB₁ at the amine group. They are N-(1-deoxy-D-fructose-1-yl)-FB₁ (NDFB₁) and N-(carboxymethyl) FB₁ (NCMFB₁). Voss et al. (2001) proved that the NDFB₁ can be created during Maillard-like reaction in the heating process. NDF was also presented when FB₁ is formed with D-glucose in the binding reaction (Poling et al., 2002). The similar process occurred in FB₂ and FB₃ with the o-phthalaldehyde (OPA) reagent (Matsuo et al., 2015). Lu et al.(2002) studied on the characterization of FB₁-glucose reaction kinetics and showed the method using glucose to decrease FB₁ concentration to half at 60 °C in 8 days or 80 °C in 2 days. In an other study, glucose was used successfully to degrade FB₁ during twin screw extrusion, till 37% (Jackson et al., 2011). N-(carboxymethyl) FB₁ was the principal reaction product following the heating process by reducing sugars in phosphate buffer in the range of 78 °C and 94 °C (Howard et al., 1998). But there is only 3 – 16% of the N-(carboxymethyl) FB₁ was created in the muffin in the extrusion conditions (Castelo et al., 2001). From the point of view of toxicity, NDF from FB₁ is less toxic than FB₁ (Voss et al., 2001).

Other functional groups which are able to affect TCAs and the metabolism products are partially hydrolysed fumonisins (PHFB) or fully hydrolysed fumonisins (HFB). The HFB₁ was determined in the 1990s (Gelderblom et al., 1993; Hopmans and Murphy, 1993; Shephard et al., 1994). This process happens during nixtamalisation using alkaline solution or processed with Ca(OH)₂ (Scott and Lawrence, 1994) or by some microorganisms (Fodor et al., 2007). Besides, several studies were conducted using enzyme Carboxylesterase FumD to degrade FB₁ into HFB₁ (Heinl et al., 2010; Hartinger et al., 2011; Masching et al., 2016). In theory, the PHFBs are easier

created than HFBs whenever the degradation process happens because of removing only one TCA group. The simpler chemical structure of HFB compared with FBs leads the higher absorption in the intestine (Caloni et al., 2002). Cirilini (2014) and coworkers reported that HFB₁ is more stable than FB₁. HFB₁ was believed more cytotoxic than FB₁ (Gelderblom et al., 1993) and HFB₁ toxicity was demonstrated in rats (Hendrich et al., 1993). But recently, the toxicity of HFBs has been investigated; they are far less toxic than FBs, especially in rodents and pigs (Collins et al., 2006; Seiferlein et al., 2007; Voss et al., 2009; Grenier et al., 2012; Harrer et al., 2013).

3.1.3. Analysis of fumonisin mycotoxins by liquid chromatography-mass spectrometry method

To quantify and qualify fumonisins in foods and feed stuff, several chromatographic methods have been developed such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) techniques. Most of the methods are applied to quantify fumonisin Bs because of its dominant presence among fumonisin analogues. Among of them LC-MS is the most frequently used method for quantification of fumonisins because of its high sensitivity and accuracy. This technique combines the physical separation capabilities of LC with the mass analytic capabilities of MS. It had been extremely difficult to connect LC with MS before the 1990s because they require rather different conditions such as temperature or volume of analytes. The atmospheric pressure ionization (API) solved effectively this problem. The atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are mainly two types of API interfaces. APCI is suitable for primarily low and medium polarity compounds whereas ESI is the best

appropriate for ionic compounds with high polarity. Therefore, ESI is selected for fumonisin determination. To analyze fumonisin isomers, LC-tandem mass spectrometry (LC-MS/MS) is usually used based on the better capability of separation and identification of compounds in complex mixtures. The analytical conditions applied to LC-MS/MS depend on the type of fumonisin and the type of samples (Table 3). The limit of quantification (LOQ) for FB₁ and FB₂ was 2 µg kg⁻¹ (D'Arco et al., 2008), while Silva et al., (2009) reported higher LOQ value, 12 µg kg⁻¹, for fumonisins B₁ and B₂, using the same LC-MS/MS system and conditions for corn-based foods analysis. Their method was modified by using ultrasonic extraction and LOQs for FB₁ and FB₂ were 11.7 µg kg⁻¹ and 8.3 µg kg⁻¹ respectively, from fresh corn samples (Li et al., 2012). In order to identify fumonisins and qualify them in corn, Tamura et al. (2015) utilized LC-Orbitrap MS. LOQs for FA₁, FA₂, FA₃ were 0.34 µg kg⁻¹, 1.98 µg kg⁻¹ and 0.92 µg kg⁻¹, respectively. LC-Orbitrap MS analysis proved to be better than LC-MS/MS regarding the detection of fumonisins at very low levels, as LOQs were between 0.05 and 0.12 µg kg⁻¹ for FBs.

Table 3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) conditions applied to the separation of fumonisins

Type of fumonisin	Samples	Instrument	The mobile phase of LC	MS/MS condition	References
FB ₁ , FB ₂ , FB ₃	Corn-based foods	LC Alliance 2695 system; TQ mass spectrometer Quattro LC from Micromass	Water + 0.5% formic acid (A) and Methanol + 0.5% formic acid (B). An isocratic step of 65% B for 3 min, gradually increased to 95% B in 4 min and held constantly for 7 min. Flow rate is 0.5 ml min ⁻¹	Positive ion mode. The (ESI) source values: capillary voltage, 3.20 kV; source temperature, 125°C; desolvation temperature: 300°C; desolvation gas: nitrogen, 99.99% purity, flow: 500 l/h.	D'Arco et al., 2008; Silva et al., 2009
FB ₁	Bovine milk	LC Alliance 2695 system; Quattro Premier XE equipped with an ESCITM Multi-Mode Ionization Source	Water:acetonitrile (90:10, v/v) + 0.3% formic acid (A) and Acetonitrile + 0.3% formic acid (B). Isocratic conditions (75%A and 25%B)	Positive ion mode. The (ESI) source values: capillary voltage: 3.25 kV; source temperature, 140°C; desolvation temperature: 400°C.	Gazzotti et al.; 2009
FB ₁ , FB ₂	Fresh corn	LC Alliance 2695 system. Waters Quattro MicroTM API triple-quadrupole MS	Methanol:water:formic acid (75:25:0.2, v/v/v)	Positive ion mode. The (ESI) source values: capillary voltage: 3.5 kV; source temperature: 120°C; desolvation temperature: 350°C. desolvation gas flow rate: 600 l/h.	Li et al., 2012

3.2. Gastrointestinal microflora in pigs, sheep and the quantitative methods

3.2.1. Gastrointestinal bacteria in pigs

The microbial ecology in the gastrointestinal tract (GIT) of pigs is complicated and the exploration is still in progress. The knowledge of the

GIT microflora is gained by the classical culturing technique in the past and the molecular biological tools nowadays. Several studies presented the classification of GIT microbial ecology by using the microbiological methods (Robinson et al., 1981 and 1984; Russel, 1979; Moore et al., 1987). As their results showed, most of bacteria are belong to the Gram-positive groups including *Streptococci* and *Clostridia* while the dominant Gram-negative bacteria are *Bacteroides*. To get more information about the GIT microbiota, the Pig Intestinal Molecular Microbiology Project was conducted by Leser and coworkers (2002). The result showed that 81% low-GC (Guanine and Cytosine) gram-positive phlotypes were represented and 11.2% belonged to *Bacteroides* and *Prevotella* group. Isaacson and Kim (2012) reviewed the component of GIT bacteria in pig and concluded that the primary (90%) of bacterial strains in the pig intestine belong to two phyla: *Firmicutes* and *Bacteroides*. By metagenomic analysis, Xiao and coworkers (2016) showed that there were 7.7 million non-redundant genes representing 719 metagenomic species of microbes in faeces from pigs. However, regarding the specific productivities of pigs such as reproduction or slaughtering, the additional feed was added in the diet to change microbial systems. For instance, antimicrobial supplements have been used to improve the body weight gain during the weaning period (Jensen, 1998; Cromwell, 2002). Dietary plant extract supplementation in weaned piglets increased the amount of *Lactobacillus* spp (Castillo et al., 2006). When Durmic et al. (1998) added resistant starch to the diet, the amount of total and Gram-negative bacteria increased in the colon.

GIT microbes play important roles in the initial colonization, the barrier function, development of the immune system and impact on feed efficiency (Fouhse et al., 2016). Most of bacteria in the pig intestine need an anaerobic

environment for existence and development which are supported by the availability of some initial colonization microbes such as *Escherichia coli* (*E. coli*) and *Streptococcus* spp. (Petri et al., 2010). A representative beneficial bacterial groups which have the protective roles and create the gut barrier are *Lactobacillus* spp. They helped mucin production (Che et al., 2014) to withstand the pathogenic microorganisms in the small intestine of the pig (Konstantinov et al., 2006). Mach et al. (2015) found the relationship between the IgA concentrations and amount of *Prevotella* while the macrophages were reduced in germ-free pigs compared with the Lactic acid bacteria (LAB) colonization group (Zhang et al., 2008). In term of feed digestion, the free amino acid can be metabolised by the luminal bacteria in the gut of the pig (Yang et al., 2014) and glutamine may relate to the process in the small intestine (Dai et al., 2012).

3.2.2. Ruminal bacteria in sheep

Ruminal microflora was researched in decades but not so many studies focused on sheep's ruminal microorganisms. In the last decades, the scientists classified bacteria into the groups of liquid-associated bacteria and solids-associated bacteria. Both of them can help the starch digestion (Faichney et al., 1997). Recently, ruminal microorganisms are usually studied by molecular biological technique. The major ruminal microorganisms of ewes belong to phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Castro-Carrera et al., 2014). Wang and coworkers (2017) used the next generation sequencing technique to analyse the bacterial system in the rumen of sheep and the results showed 133 genera divided into 16 phyla dominating with *Firmicutes* and *Bacteroidetes*. When the nutrient levels were up, *Bacteroidetes* and *Proteobacteria* increased while *Firmicutes* decreased. The construction of bacterial communities is various in a different host

(Henderson et al., 2015). In sheep, the results showed the dominance of unclassified *Veillonellaceae* while *Fibrobacter* was less than in bovines.

The ecosystem of microorganisms in the sheep rumen are still not fully understood but some isolated strains were studied on their roles in the feed digestion. In those studies, the isolates closing to *Streptococcus gallolyticus* presented the ability as the tannins resistant bacteria (Babaei et al., 2015). The sheep's ruminal anaerobic bacteria can degrade protein via the fermentation process (Ali et al., 2009). The fermenting bacterial communities such as lactate and succinate producing bacteria are also related to the lower CH₄ yields by less hydrogen digestion (Kittelman et al., 2014). Another important role of the ruminal bacteria is supported to produce NH₃ by deamination of amino acids (Eschenlauer et al., 2002). Koike and coworkers (2010) isolated successfully some fibre-associated bacteria which can degrade fibrin sheep rumen, namely B76 and R-25. Both of them were gram-positive short rods or cocci and showed hemicellulolytic activity. By pyrosequencing analysis, Wang et al. (2017) showed the entire GIT bacteria of sheep, the most dominant bacteria in sheep's rumen is *Firmicutes* and the second biggest amount of bacteria is *Bacteroidetes*.

3.2.3. Molecular tools for quantitative investigation of gastrointestinal bacteria in pigs and sheep

To measure the amount of gastrointestinal bacteria, the most popular methods are the classical culture-dependent methods. These methods use the series of 10-fold dilution of the samples. The selective media will be used for each type of bacteria with the appropriate conditions (temperature, time, anaerobic or aerobic environment) in the technique known as plate count agar (Buchbinder et al., 1951; Wehr et al., 2004). Other culture-dependent method for a scan the amount of bacteria is the most probable number (MPN) which

use the system of cultural tubes and get the quantitative results by comparing the picture of the sample to the standard (Oblinger and Koburger, 1975). The advantage of culturing methods is to know the number of culturable and alive bacteria. However, the biggest problem is that there is over 99% of the microorganisms are not cultivable by those techniques (Hugenholtz et al., 2002). Culture-independent analysis of bacteria was developed based on the specification of the bacterial genes. The method using 16S rRNA gene was applied to investigate the composition of intestinal microorganisms (Amann et al., 1995; Kageyama et al., 2000; Sakamoto et al., 2000). Up to now, there are several molecular methods have been used to determine the qualitative (types of bacteria) and quantitative (amount of a given bacteria) properties of GIT bacteria such as real-time PCR (or qPCR), next generation sequencing (NGS) and the phylochip.

Real-time PCR is a PCR which can monitor the amplification process of a target gene during the PCR. To track the PCR product, the fluorescent dyes or labelled DNA probes bind the DNA, which the fluorescent dye/label is detected during each cycle of the amplification. This technique has been selected by many types of research because of the specific, accurate and reasonable cost assay. The amount of the total bacteria (11.1 ± 0.88 log gene copy number/g fresh matter (FM)), *Lactobacilli* (7.8 ± 0.37) and *Enterobacteria* (10.8 ± 1.66) in the jejunum of pigs were measured by quantitative real-time PCR (qPCR) (Castillo et al., 2006). Okabe and coworkers (2007) set up the qPCR for investigating faecal pollution (from human, cow and pig) in freshwater base on the host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers. The qPCR has been also applied to determine the amount of ruminal bacteria and the result showed that *Fibrobacter succinogenes* were the most abundant species. In sheep's rumen, Mosoni et al. (2007) quantified *Fibrobacter succinogenes*, *Ruminococcus*

albus and *Ruminococcus flavefaciens* by qPCR targeting 16S rRNA and reported that *R. albus* was presented at the lowest amount.

There are different approaches to perform NGS. In most cases, NGS is started with the preparation of DNA fragmentation library and *in vitro* adaptor ligation. The second step is divided into two types of PCR, the bridge PCR which is applied for sequencing by synthesis and the emulsion PCR which is used in pyrosequencing and sequencing by ligation. The NGS can show the sequences of the microflora population and their amount in each phyla. Pajarillo and coworker (2014) have been analysed the fecal microflora of Duroc pigs by pyrosequencing and showed the dominance of *Prevotella* as well as a phylotype similar to *Oscillibacter valericigenes*. In the latest investigation of bacteria in the stomach of pig by 16S rRNA analysis using the NGS, Motta et al. (2017) reported that *Proteobacteria* was the dominant phylum in the gastric contents while the bacteria of the gastric mucus belonged to *Herbiconiux* and *Brevundimonas*. The characterization of the microbial communities in the GIT tract of sheep, from the rumen to rectum, were studied by Wang and coworkers (2017) using 454 pyrosequencing analysis. The result showed the various phyla depending on the different part of the GIT but the dominant bacterial phyla in the entire sections were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*.

The phylochip or different microarrays are working with probes attached to a solid surface. The samples find the probes, then the signal will be detected and analysed by the reader or by a computer program depending on the probe density on the surface. Maga et al. (2013) used pigs as the animal model to study composition of gut microbiota. The phylochip was utilized as the analytic method and the result showed that two major phyla of the faeces during milk supplementation are *Bacteroides* and *Firmicutes*. To analyse ruminal bacterial communities, the first phylochip was studied and developed

by Kim and coworkers (2014) and it is designed to detect 1666 operational taxonomic units.

3.3. Interactions between fumonisins and bacteria

3.3.1. Effect of fumonisins on bacteria

Fumonisins might affect bacterial activities directly or indirectly. However, the scientific information about the impact of fumonisins on bacterial activities is very sparse. To the best of our knowledge, there was only one report from Becker (1997) about the direct effect of fumonisins on some certain bacteria strains. Some *in vitro* experiments were conducted to estimate the influence of FB₁ on the bacteria isolated from various sources. As the results showed, a large amount of bacteria did not differ significantly between control and treated groups (the range of fumonisin concentration was from 50 µM to 1000 µM) after incubation (Becker et al., 1997). By contrast, some positive results about the indirect impact of fumonisins on bacteria were published. Activities of pathogenic bacteria and the immune system of the body have a tight connection. Fumonisins, which cause immunotoxicity in mice (Abbès et al., 2015) and reduce the phagocytic activity of chicken macrophages (Chatterjee and Mukherjee, 1994), can influence activities of colonized bacteria in the body. When Japanese quails were infected with *Salmonella gallinarum*, increased mortality and decreased lymphocyte number was observed in FB₁ treated group at 150 mg/kg feed for 6 weeks (Deshmukh et al., 2005). Colonization of the small and large intestines by an extra intestinal pathogenic *E.coli* strain was significantly proliferated when pigs were treated with 0.5 mg/kg of body weight of FB₁ for 7 days (Oswald et al., 2003). In case of co-occurrence of fumonisins and other mycotoxins, especially aflatoxin, also intensified the calf susceptibility to Shiga toxin or verotoxin producing *E. coli* (STEC) associated with

hemorrhagic enteritis (Baines et al., 2013). Burel et al. (2013) reported that chronic exposure to a medium concentration of fumonisins in the naturally contaminated feed (11.8 mg/kg for 63 days) had no effect on the pigs' health but could affect the balance of the microbiota.

3.3.2. Effect of bacteria on fumonisins

There are reported impacts of bacteria on *Fusarium* mycotoxins and fumonisins. Bacteria can metabolize or bind fumonisins directly or inhibit fumonisins production of fungi (Table 4).

As for inhibition of fumonisins, *Lactobacillus rhamnosus* can inhibit FB₁ production in the range from 78.64% to 92.88% efficiency and significantly reduces bad impacts of FB₁ to liver and kidney of the rat. (Al-Masri et al., 2011). This bacterial strain can diminish FB₂ production up to 43.4% in experimental groups (Stiles and Bullerman, 2002). Some isolated rhizobacteria strains were demonstrated to have biological effects on *Fusarium verticillioides* and FB₁. In these bacterial groups, *Pseudomonas solanacearum* and *Bacillus subtilis* strongly inhibited FB₁ production in the range from 70% to 100% (Cavaglieri et al., 2005; Formenti et al., 2012). The concentration of FB₁ was reduced by *Lactobacillus subsp. paracasei* after 20-day incubation (70.5 µl/ml compared with 300 µl/ml FB₁ in control group) and *Lactobacillus paracasei subsp. Paracasei* can inhibit FB₁ production in 10-day incubation (Gomah and Zohri, 2014). In an other report, FB₁ level in maize was decreased by lactic acid bacterial activity after 3-day fermentation (Mokoena et al., 2005). A significant reduction of FB₁ production and growth of *Fusarium verticillioides* was reported when cultured with *Propionibacterium freudenreichii* ssp. *shermanii* and ssp. *Freudenreichii* (Gwiazdowska et al., 2008). Concentration of FB₁ and FB₂ were also reduced significantly by *Bacillus amyloliquefaciens*, *Microbacterium oleovorans* and *Enterobacter hormaechei*

(Pereira et al., 2007; Pereira et al., 2010; Sartori et al., 2013). FB₁ was hydrolyzed and deaminated by a bacterial strain isolated from soil after 3 hour period of incubation. There was a close phylogenetic relationship between this bacterium and the *Delftia acidovorans* as well as *Comamonas* group (Benedetti et al., 2006). FB₁ was also degraded by *Bacillus sp.* isolated from corn and silage in the range from 43% to 83% after 6-day incubation. Based on the results of Takeuchi et al. (2001) and Täubel (2005), two bacterial enzymes from *Sphingopyxis sp. MTA144* were demonstrated that they can degrade FB₁ (Heinl et al., 2010). Some bacteria can bind fumonisins, Niderkorn (2006) reported that 82% FB₁ can be removed by *Leuconostoc mesenteroides* and 100% FB₂ can be eliminated by *Lactococcus lactis*. *Streptococcus* and *Enterococcus* also have a significant effect on FB₁ and FB₂ level, these bacteria bind FB₁ and FB₂ up to 24 and 62%, respectively (Niderkorn et al., 2007).

Table 4. Effect of bacteria on fumonisins

Microorganism	Effect on fumonisins	Period of time	Level of effect	Sources
<i>Lactobacillus subsp. Paracasei</i>	Inhibition of FB ₁ production	28°C / 20 days	76.5%	Gomah and Zohri, 2014
<i>Pseudomonas solanacearum</i>	Inhibition of FB ₁ production	25°C / 20 days	100%	Cavaglieri et al., 2004
<i>Bacillus subtilis</i>	Inhibition of FB ₁ production	25°C / 20 days	70% -100%	Cavaglieri et al., 2004
Bacterial strains isolated from soil (close with <i>Delftia acidovorans</i> and <i>Comamonas</i>)	Hydrolysis and deamination of FB ₁	25°C / 3 hours	100%	Benedetti et al., 2006
<i>Bacillus sp.</i>	Degradation of FB ₁	35°C / 6 days	43% - 83%	Camilo et al., 2000
<i>Streptococcus thermophilus</i>	Binding FB ₁ and FB ₂	25°C / 24 hours	24% and 62%	Niderkorn et al., 2007
<i>Enterococcus</i>	Binding FB ₁ and FB ₂	25°C / 24 hours	14% and 43%	Niderkorn et al., 2007
<i>Leuconostoc mesenteroides</i>	Elimination of FB ₁	30°C / 24 hours	82%	Niderkorn et al., 2006
<i>Lactococcus lactis</i> .	Elimination of FB ₂	30°C / 24 hours	100%	Niderkorn et al., 2006

4. Objectives of the dissertation

The aim of the research was to determine the interaction between fumonisins and the gastrointestinal microbiota from the following aspects:

1 - The effect of fumonisin mycotoxin on the bacterial communities of the gastrointestinal tract in sheep and swine.

In pigs, the *in vitro* and *in vivo* experiments were performed and the amount of caecal bacteria were measured by culturing technique and qPCR.

In sheep, an *in vitro* experiment using quantitative polymerase chain reaction (qPCR) was designed to determine DNA copy numbers of ruminal bacteria.

2 - The effect of the microorganisms of the gastrointestinal tract of swine on the metabolism of fumonisin.

5. Materials and methods

Three experiments were performed in this research and the methodologies were presented briefly as below:

Experiment 1: *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

Methods: Liquid chromatography-mass spectrometry (LC-MS), plate count agar technique, quantitative polymerase chain reaction (qPCR)

Experiment 2: *In vitro* effect of fumonisin B₁ on the ruminal microflora of sheep

Methods: qPCR

Experiment 3: *In vivo* experiment: Effect of fumonisins producing *Fusarium sp.* on the microbiota in pigs

Methods: Plate count agar technique, qPCR

5.1. Experimental designs

5.1.1. *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

Samples of caecal content were collected from adult pigs (n=2; Hungarian Large White) right after slaughtering in 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 (24h/37°C/anaerobic) McDougall buffer solution (9.8 g NaHCO₃, 3.7 g anhydrous Na₂HPO₄, 0.57 g KCl, 0.47 g NaCl, 0.12 g MgSO₄.7H₂O, 0.04 g CaCl₂ and 1000 ml aquadest; pH 8.3) were prepared to serve as a control solution and to homogenise samples.

The experiment consisted of 3 groups as shown in Table 5. Two control groups were set up. Tubes in the control 1 group contained buffer and chyme,

while control 2 group was prepared including buffer and FB₁.

Caecal chyme was homogenised and divided into the control 1 and experimental groups. An aliquot of 3.33g of caecal chyme was suspended in pre-incubated McDougal buffer tubes (experimental and the control 1 group). After a pre-incubation for four hours at 37°C, FB₁ (50 µg/g; Sigma-Aldrich, Darmstadt, Germany) was added to each tube (experimental and control 2 groups) to get a final concentration of 5µg/ml. Samples were taken at 0, 24 and 48 h of anaerobic incubation for determination of bacterial numbers and FB₁ concentration.

Table 5: Experimental design to determine *in vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

Incubation (h)/treatment	Experimental group (Buffer+Chyme+FB₁)	Control 1 group (Buffer+Chyme)	Control 2 group (Buffer+FB₁)
0 h	4	4	4
24 h	4	4	4
48 h	4	4	4
Description	12 x 3,33 g chyme 12 x 5,67 ml buffer 12 x 1 ml 50 µg/g FB ₁	12 x 3,33 g chyme 12 x 5,67 ml buffer 1 ml H ₂ O	12 x 9 ml buffer 12 x 1 ml 50 µg/g FB ₁

5.1.2. In vitro effect of fumonisin B₁ on the ruminal microflora of sheep

Samples of ruminal content were collected from adult sheep (n=2; Racka and Merino crossbred) right after slaughtering in a slaughter house and transferred into sterile bottles. The bottles were put in anaerobic plastic bags with an Anaerocult gas generator (Merck, Darmstadt, Germany). The pre-incubated (24h/37 °C/anaerobic) McDougall buffer solution was prepared to homogenise samples and make the solution for control groups.

The experiment was designed to have an experimental group and a control group as shown in Table 6. Tubes in control contained buffer and ruminal

content, while the experimental group was prepared including buffer, ruminal content and FB₁.

The ruminal content was homogenised and divided into control and experimental groups. 3.33g of ruminal content was suspended in pre-incubated McDougal buffer tubes. After a pre-incubation for four hours at 37 °C, FB₁ (50 µg g⁻¹; Sigma-Aldrich, Darmstadt, Germany) was added to each experimental tube to get a final concentration of 5µg ml⁻¹. Samples were taken at 0, 24 and 48 h of anaerobic incubation for determination of bacterial numbers.

Table 6: Experimental design to determine *in vitro* interaction between fumonisin B₁ and the intestinal microflora of sheep

Incubation (h)/treatment	Experimental group (Buffer+Ruminal content+FB ₁)	Control group (Buffer+ Ruminal content)
0 h	4	4
24 h	4	4
48 h	4	4
Description	12 x 3,33 g ruminal content 12 x 5,67 ml buffer 12 x 1 ml 50 µg g ⁻¹ FB ₁	12 x 3,33 g ruminal content 12 x 5,67 ml buffer 1 ml H ₂ O

5.1.3. In vivo experiment: Effect of Fumonisin producing Fusarium sp. on the microbiota in pigs

5.1.3.1. Experimental animals and the design of the experiment

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

Twelve weaned barrows (n=12) of the same genotype, weighing 12–14 kg, were used in the experiment. The piglets were weighed and then divided into two groups: an experimental group (n = 6) and a control group (n = 6). The animals were placed into metabolic cages (80 x 80 cm) during the trial. The

temperature of the trial room were controlled in accordance with the needs of weaned piglets. Feed was given twice a day, in two equal portions, and the amount of feed not consumed by the animals were measured back. Drinking water was available *ad libitum* via automatic drinkers. After a 7-day adaptation period, according to the method of Tossenberger et al. (2000), a T-cannula were implanted into the caecum, in order to determine the effect of FB₁ on the microbiota of the caecum. The duration of the trial was 9 days (following a 7-day adaptation phase and a 1-week regeneration phase after the operation). The experimental animals were fed a basic ration of a composition corresponding to their age (400 – 500 gram/day/pig). After the regeneration period, a *Fusarium verticillioides* fungal culture was mixed into the ration of the experimental animals, so as to provide a daily FB₁ intake of 10 mg/animal. The mycotoxin content (Zearalenone (ZEN), Deoxynivalenol (DON), Fumonisin B₁) of the control and experimental feed was measured.

5.1.3.2. Sampling and processing

In the period of *Fusarium verticillioides* feeding (9 days) samples of caecal content were taken on days 0, 2, 4, 6 and 8 through the T-cannula and transferred into sterile tubes. The tubes of samples on days 0, 4 and 8 were prepared for microbial culturing. 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. Samples from all tubes in the collecting points of time were stored in deep freezer (-86 °C) for qPCR analysis.

5.2. Measurement of the amount of bacteria

5.2.1. Media and plate count agar technique applying to measure living bacteria in the in vitro and in vivo experiment in pigs

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.

The 10-folds series dilution was conducted from 10^{-1} to 10^{-8} . An aliquot (100 μ l) was pipetted and added to the surface of each respective selected agar to culture bacteria. Five groups of bacteria were enumerated, in the *in vitro* experiment in pigs, including aerobic bacteria, anaerobic bacteria, coliform, *Escherichia coli* (*E. coli*) and *Lactobacillus sp.* while in the *in vivo* experiment, one additional bacterial strain was counted, besides the above mentioned five groups, *Clostridium perfringens* (*C. perfringens*). The aerobic and anaerobic bacteria were cultured in commercial blood agar (BA; Bakt-Teszt Ltd., Budapest, Hungary). Coliform and *Escherichia coli* population were estimated on ChromoBio Coliform Agar (BioLab). The amount of *Lactobacillus sp.* was determined by using MRS agar (BioLab). For enumeration of *C. perfringens*, the pour plating technique with Tryptose sulphite cycloserine (TSC) agar (ISO7937 – VWR Chemical) was applied. The same amount of diluted sample (100 μ l) was pipetted and mixed with TSC agar (10 ml) on the petri dish. Then the other 10 ml TSC agar was utilised to cover thick layer after complete solidification of the previous medium.

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

$$N = \frac{\Sigma C}{V \times 1,1 \times d} \quad \text{[Equation 1.]}$$

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; ‘d’ is the dilution corresponding to the first dilution retained.

5.2.2. Quantitative polymerase chain reaction (qPCR) applying to measure DNA copy numbers of bacteria

The qPCRs performed to investigate DNA copy numbers of bacteria in all experiments in this research are summarised in Table 7

**Table 7. Bacteria groups investigated in the research
for a description of Exp. 1-3 see the beginning of Section 5**

Number	Bacteria	Exp. 1	Exp. 2	Exp. 3
1	Total bacteria	X	X	X
2	<i>E. coli</i>			X
3	<i>Enterobacteria</i>			X
4	<i>Bacteroides</i> and <i>Prevotella</i>	X	X	X
5	<i>Clostridium sp.</i>			X
6	<i>Lactobacillus sp.</i>	X		X
7	<i>Firmicutes</i>		X	X
8	<i>Delta-</i> and <i>Gammaproteobacteria</i>		X	

Exp. : Experiment

DNA extraction and QPCR

The DNA extraction was carried out with approximately 200 mg of the frozen samples using the QIAamp®DNA Stool Mini Kit according to the manufacturer's instructions.

The standard curve was created by dilution series of purified PCR products for *Lactobacillus sp.*, *Firmicutes*, *Delta-* and *Gammaproteobacteria* whereas the dilution series of plasmid concentration was used to prepare the standard curve for total bacteria, *E.coli*, *Enterobacteria*, *C. perfringens*, *Bacteroides* and *Prevotella*.

The quantity of bacterial groups was determined by qPCR using SYBR Green. The primers for investigated bacterial groups were selected based on previous literature (Table 8). 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 each primer, 10.5 µl sterile DEPC treated distilled water and 1 µl of DNA extract. The PCR program for total bacteria, *Enterobacteria*, *E.coli*, *Bacteroides* and *Prevotella* consisted of 10 min at 95 °C, 40 cycles of 30 sec at 95 °C, 1 min at 60 °C. The PCR program for *Firmicutes*, *Delta-* and *Gammaproteobacteria* was 10 min at 95

°C, 40 cycles of 15 sec at 95 °C, 1 min at 60 °C. To investigate the amount of *Clostridium sp.*, the PCR program was 3 min at 95 °C, 40 cycles of 40 sec at 95 °C, 40 sec at 54 °C, 80 sec 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 one gram of sample contents.

Table 8. Oligonucleotide sequences used for QPCRs

Investigated group	Oligonucleotide sequence (5'–3')	Amp. (bp)	References
Total bacteria	Forward: GCAGGCCTAACACATGCAAGTC Reverse: CTGCTGCCTCCCGTAGGAGT	292	Amann et al. (1995); Marchesi et al. (1998); Castillo et al. (2006)
<i>Enterobacteria</i>	Forward: ATGGCTGTCGTCAGCTCGT Reverse: CCTACTTCTTTTGCAACCCACTC	177	Sghir et al. (2000); Leser et al. (2002); Castillo et al. (2006)
<i>Escherichia coli</i>	Forward: GGTATGGATCGTTCGACCT Reverse: GGCAGAATGGTAACACCAGAGT	300	Banu et al. (2010); Pers-Kamczyc et al. (2011)
<i>Bacteroides and Prevotella</i>	Forward: GAAGGTCCCCCACATTG Reverse: CAATCGGAGTTCTTCGTG	418	Kim 2011
<i>Clostridium sp.</i>	Forward: AAAGGAAGATTAATACCGCATAA Reverse: ATCTTGCGACCGTACTCCCC	722	Mirhosseini et al. 2010
<i>Lactobacillus sp.</i>	Forward: AGCAGTAGGGAATCTTCCA Reverse: CACCGCTACACATGGAG	340	Walter et al. (2000) Heilig et al. (2002); Su et al. (2008)
<i>Firmicutes sp.</i>	Forward: GGAGYATGTGGTTTAATTCGAAGCA Reverse: AGCTGACGACAACCATGCAC	126	Guo et al. (2008)
<i>Delta - and Gammaproteobacteria</i>	Forward:GCTAACGCATTAAGTRYCCC G Reverse: GCCATGCRGCACCTGTCT	189	Yang et al. (2015)

Amp.: Amplicon

5.3. Mycotoxin extraction and analysis

For FB₁ extraction, the post-incubated samples from the experimental group and the control-2 group were diluted 2-fold (7 ml sample and 7 ml distilled water) and centrifuged for 5 minutes (3000 rpm). The supernatant was used for FB₁ extraction followed by the modified protocol of Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA) (Szabó-Fodor et al., 2014). The column preconditioning was conducted with 2 ml of methanol then 2 ml of distilled water. The diluted sample (2 ml) was subsequently loaded onto the columns then washed again with 2 ml of distilled water. The elution of FB₁ 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 2.5 μ C18(2)-HST column (100 mm \times 2.00 mm). The column temperature was set to 40 °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 acetonitrile (eluent B), both acidified with 0.1% acetic acid. 10 μ l of each sample 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 2,5 minicolumn re-equilibration at eluent 5% B. FB₁ (diluted from 1000 mg/l) and HFB₁ (diluted from 25 mg/l) standard solutions used as references. MS parameters: source block temperature 90 °C; desolvation temperature 250 °C; heat block temperature 200 °C; drying gas flow 15.0 l/minute. Detection was performed

using selected ion monitoring (SIM) mode.

The efficiency of FB₁ conversion to fully hydrolysed FB₁ (HFB₁) was calculated on the basis of the molecular weight of the compounds (FB₁: 721 g/mol; HFB₁: 405 g/mol) and described as below:

$$\frac{\text{Hydrolysed fumonisin B}_1 \text{ (mol/g)} \times 721 \text{ g/mol}}{405 \text{ g/mol} \times \text{Fumonisin B}_1 \text{ (mol/g)}} \quad [\text{Equation 2.}]$$

5.4. Statistical analysis

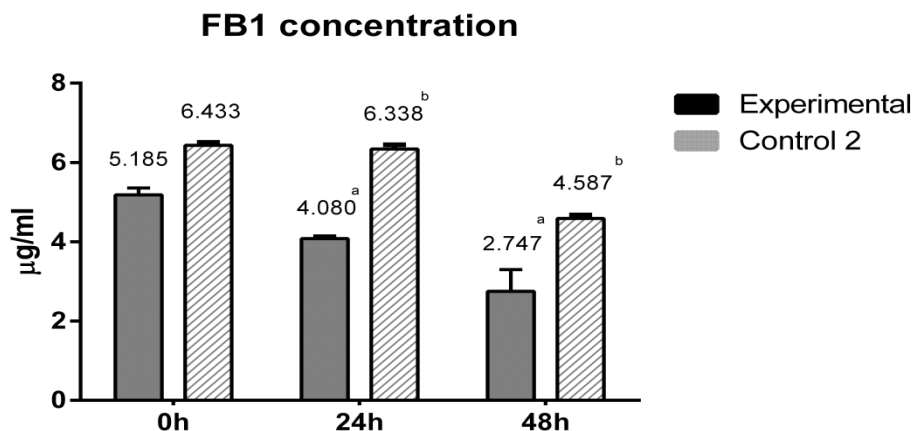
The R i386 3.1.2 program and the IBM SPSS 22 program were applied for statistical analyses. The comparative means were performed by Independent Samples t-Test, oneway ANOVA with Tukey post-hoc test and non-parametric Kruskal-Wallis test if the normal distribution was not presented. The Repeated measures ANOVA was used to analyse the colony forming units (CFUs) as well as the amount of bacterial DNA copy number during the incubation time.

6. Results and the evaluation

6.1. *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

6.1.1. Effect of caecal microflora on fumonisin B₁

At the 0 h incubation time, no significant FB₁ concentration difference between the experimental group (buffer, caecal content, FB₁) and control 2 groups (buffer, FB₁) was observed; 5.185 ± 0.175 $\mu\text{g/ml}$ compared with 6.433 ± 0.076 $\mu\text{g/ml}$, respectively. FB₁ concentration in experimental groups was significantly lower than control-2 group after 24 h and 48 h incubation period, 4.080 ± 0.065 $\mu\text{g/ml}$ and 2.747 ± 0.548 $\mu\text{g/ml}$ compared to 6.338 ± 0.108 $\mu\text{g/ml}$ and 4.587 ± 0.085 $\mu\text{g/ml}$, respectively. FB₁ concentration also decreased during incubation time in the experimental group (Figure 2). HFB₁ concentration has also been determined at different incubation times. Due to the appearance of the main products of the metabolism (HFB₁) only in the experimental group (Figure 3), we can conclude that FB₁ may be metabolised by microbiota in the caecum of the pig.



^{a, b} significant ($P < 0.05$) difference between both groups

Figure 2. Fumonisin B₁ concentration in experimental groups and control 2 groups during the incubation time

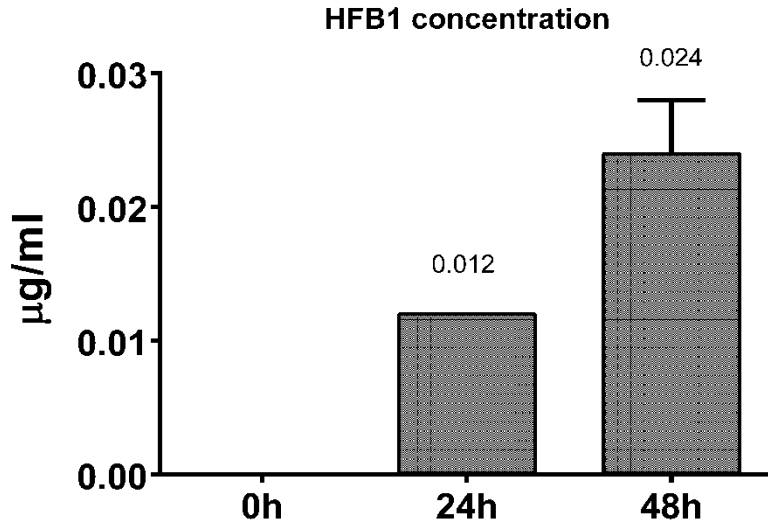


Figure 3. Hydrolysed Fumonisin B₁ concentration in experimental groups during the incubation time

The capability of bacteria to influence fumonisins was proven (Niderkorn et al., 2009; Zoghi et al., 2014). Peptidoglycan, the component of the bacterial cell wall, plays a crucial role to bind many mycotoxins including fumonisins. *Lactobacillus sp.* is the class of bacteria having a significant impact on fumonisins. The FB₁ 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 on fumonisins, most of the studies were conducted to estimate the impact of bacteria on fumonisin produced by *Fusarium sp.* such as binding or inhibition of fumonisin production while few of them have concerned about fumonisin metabolism. The concentration of FB₁ was reduced by *Lactobacillus paracasei* subsp. *Paracasei* after 20-day incubation (70.5 µl/ml compared with 300 µl/ml FB₁ in the control group) and *Lactobacillus paracasei* subsp. *Paracasei* could inhibit FB₁ production in a 10-day incubation period (Gomah and Zohri, 2014). Becker et al., (1997) reported that FB₁ was not degraded by *Enterococcus faecium* while the

binding of FB₁ and FB₂, 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 FB₁ to HFB₁ was less than 1% where there was no change in the degree of the conversion of FB₁ to aminopentol (fully hydrolysed FB₁). In this study, conversion of FB₁ to HFB₁ increased significantly from 0.33% to 0.66% after 24 h and 48 h incubation time, respectively. Differences in the HFB₁ 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).

6.1.2. Effect of fumonisin B₁ 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 FB₁ during the period of the incubation time except the group of anaerobic bacteria. The log₁₀ number of anaerobic bacteria decreased from 9.046 ± 0.036 (0 h incubation) to 8.389 ± 0.143 (48 h incubation) (Table 9). In the caecal bacteria with FB₁ groups, reduction of the log₁₀ number of anaerobic bacteria was 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. Nonetheless, there was no detectable change in microbial culturing method between the groups of caecal bacteria with and without FB₁ during the incubation time.

The quantitative PCR was also performed to determine the effect of FB₁ on Total bacteria, *Bacteroides* and *Prevotella* and *Lactobacillus sp.* The log₁₀

copy-numbers were applied for data analysis (Table 10). The \log_{10} of *Lactobacillus*, *Bacteroides* and *Prevotella* in control 1 and experimental groups augmented after 24 h incubation ($P < 0.05$). A number of Total bacteria were stable during the incubation time in the control groups while there was an increase in the experimental group from 11.520 at the 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. FB₁ did not affect the number of caecal bacteria in pigs.

As we have detected both in the microbial culture and in a qPCR experiment during the incubation time, the anaerobic bacteria decreased while the amount of *Lactobacillus sp.* increased. According to qPCR results, amount of *Bacteroides* and *Prevotella* has also increased. The primary difference between the results of two methods is that anaerobic bacteria enumerating by culture is based on the number of 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. Next experiments should be focused on other kinds of anaerobic bacteria or all bacterial species using next generation sequencing approach.

To the best of our knowledge, there was no completed 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 FB₁ (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 FB₁ (Knasmüller et al., 1997) and the number of *E.coli* showed no change in the presence of FB₁ in this study. However, the intestinal colonisation by pathogenic *E.coli* in pigs treated FB₁

was strengthened in an *in vivo* experiment (Oswald et al., 2003). The indirect impact of fumonisin on bacteria was also demonstrated in some documents; e.g. immune suppressive effects and decrease of 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).

Table 9: Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B₁ measured by culturing (log₁₀ CFU¹/g, means ± SD)

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

¹CFU: colony forming unit

^{a, b, c} significant (P < 0.01) difference between incubation times within groups.

Exp. group: Experimental group

Table 10: Number of bacteria in the pigs' caecal chyme incubated with (experimental group) and without (control 1 group) fumonisin B₁ measured by qPCR (log₁₀ copy number/g, means ± SD)

Bacteria	Incubation time					
	0 hour		24 hour		48 hour	
	Control 1	Exp. group	Control 1	Exp. group	Control 1	Exp. group
Total bacteria	11.33 ± 0.38	11.52 ^a ± 0.16	11.68 ± 0.21	11.91 ^b ± 0.03	11.66 ± 0.13	11.79 ^b ± 0.05
<i>Bacteroides</i> and <i>Prevotella</i>	7.32 ^a ± 0.28	7.41 ^a ± 0.14	7.95 ^b ± 0.16	7.83 ^b ± 0.13	7.83 ^b ± 0.12	7.97 ^b ± 0.11
<i>Lactobacillus</i>	9.61 ^a ± 0.40	9.80 ^a ± 0.25	11.35 ^b ± 0.11	11.23 ^b ± 0.17	11.13 ^b ± 0.15	11.33 ^b ± 0.14

^{a, b} significant (P < 0.05) difference between incubation times within groups.

Exp. group: Experimental group

6.2. *In vitro* effect of fumonisin B₁ on ruminal microbiota of sheep

In sheep's rumen, *Firmicutes* and *Bacteroidetes* were the most predominant bacterial phyla (Omoniyi et al., 2014). Besides, *Proteobacteria* is also the popular genera in all gastrointestinal tract of sheep (Stiverson et al., 2011; Wang et al., 2016). In this study, four groups of bacteria were quantitatively determined by quantitative PCR including total bacteria, *Bacteroides* and *Prevotella*, *Firmicutes*, *Delta*- and *Gammaproteobacteria*. The log₁₀ copy-numbers were applied for data analysis (Table 11). The growth of bacterial groups during the incubation time was analysed by one-way ANOVA. In control groups, the total bacteria and *Delta*- and *Gammaproteobacteria* was stable ($P>0.05$) while the significant changes of *Firmicutes*, *Bacteroides* and *Prevotella* were observed ($P<0.05$). In the experimental group, only total bacteria was keeping stability in the entire experimental incubation time.

Regarding the differences between control-1 and experimental group, total bacteria, *Firmicutes* and *Delta-Gammaproteobacteria* DNA copy number, none of their tested time points changed while the *Bacteroides* and *Prevotella* group presented significant differences after 24 and 48 hour incubation, 8.36 ± 0.07 and 7.73 ± 0.04 compared with 8.48 ± 0.05 and 8.04 ± 0.16 , respectively. In total, the repeated measures ANOVA was applied to analyse the data and the trends of bacterial growth were compared. Statistically significant difference was observed between the control and experimental group in *Bacteroides* and *Prevotella* whereas no change was observed in the remaining investigated bacterial groups. FB₁ had affected the number of *Bacteroides* and *Prevotella* and the values of data showed that the amount of those bacteria in the experimental group was higher than the ones in the control group. There is no information about the FB₁ consuming capability of *Bacteroides* and *Prevotella*. So we assume that other types of the bacterial group have been decreased then *Bacteroides* and *Prevotella* grew for keeping balance in the total bacterial communities. Other types of bacteria should be examined to understand the

phenomenon. Srichana et al. (2009) used culture optical density (OD) to estimate the ruminal bacteria population when the microbes treated with fumonisin. They reported that the OD of the fumonisin mixed group (100 µg/ml and 200 µg/ml) were significantly higher than the OD of the group without fumonisin, 1.66 and 1.62 compared with 1.41, respectively. Up to now, there had been no report of the impact of FB₁ on sheep's ruminal bacteria. The further experiment should be conducted to gain more information on this issue.

Table 11: Number of bacteria in the sheep's ruminal content incubated with (experimental group) and without (control 1 group) fumonisin B₁ measured by qPCR (log₁₀ copy number/g, means ± SD)

Bacteria	Incubation time					
	0 hour		24 hour		48 hour	
	Control	Experiment	Control	Experiment	Control	Experiment
Total bacteria	11.05 ± 0.12	11.14 ± 0.04	11.16 ± 0.17	11.13 ± 0.55	11.11 ± 0.02	11.11 ± 0.12
<i>Bacteroides</i> and <i>Prevotella</i>	8.19 ± 0.03	8.22 ± 0.03	8.36 ^a ± 0.07	8.48 ^b ± 0.05	7.73 ^a ± 0.04	8.04 ^b ± 0.16
<i>Firmicutes</i>	8.66 ± 0.04	8.71 ± 0.05	8.77 ± 0.11	8.85 ± 0.03	8.55 ± 0.06	8.52 ± 0.10
<i>Delta</i> -and <i>Gammaproteobacteria</i>	5.95 ± 0.09	6.00 ± 0.06	6.02 ± 0.13	6.15 ± 0.08	5.97 ± 0.10	5.95 ± 0.13

^{a, b} significant (P < 0.05) difference between both groups

6.3. *In vivo* experiment: Effect of fumonisins producing *Fusarium sp.* on the microbiota in pigs

The alteration of the amount of living bacteria in the pigs' caecum showed in Table 12. Six bacterial types were investigated including aerobe, anaerobe, *E.coli*, Coliforms, *Lactobacillus sp.* and *C. perfringens*. Only one slight difference was observed between the aerobe of control and experimental groups at Day_4, 8.60 ± 0.22 compared with 8.06 ± 0.20 (P < 0.05), respectively but there was no change during the trial within each group as well as in trending comparison between two groups. The number of anaerobe bacterial species increased while the amount of *C. perfringens* decreased during the time (P < 0.05) within each group, control and experiment. However, no differences were presented in the entire comparison between *Fusarium* and no *Fusarium* feeding groups. There was no significant

change in the amount of *E.coli*, *Coliform* and *Lactobacillus sp.* in all sampling points of time.

Most of the bacterial species in the gastrointestinal tract can not be identified by culturing but by genetic tools. In the intestine of a pig, *Firmicutes* and *Bacteroidetes* are the most dominant phylum (Isaacson and Kim, 2012). *Firmicutes* are the huge phylum major covering Gram-positive bacteria such as *Bacilli*, *Clostridia* and *Erysipelotrichia* whereas *Bacteroidetes* consists many classes of Gram-negative bacteria including *Bacteroides* and *Prevotella*. Besides those big phyla, other types of bacteria were investigated by qPCR in this study such as *Enterobacteria* and *E.coli* (Table 13). The amount of total bacteria was altered within each group ($P < 0.01$) and the significant differences were observed at some sampling points of time, Day_2 and Day_6. Considerable differences between control and experimental groups were presented in *Firmicutes* at Day_2, *Enterobacteria* and *E.coli* at Day_4. The number of scanned bacterial species was changed during feeding time. However, there was not a significant difference in the entire comparison of all investigated bacteria between the control and experimental groups.

Table 12: Number of bacteria in the pigs' caecal chyme with (experimental group) and without (control group) fumonisin B₁ measured by culturing (log₁₀ CFU¹/g, means ± SD)

Groups	Period of the feeding time					
	Day0		Day4		Day8	
	C	E	C	E	C	E
Aerobe	8.44 ± 0.10	8.06 ± 0.41	8.60 ^b ± 0.22	8.06 ^a ± 0.20	8.56 ± 0.48	8.13 ± 0.62
Anaerobe	8.65 ± 0.07	8.68 ± 0.35	9.36 ± 0.33	9.26 ± 0.17	9.42 ± 0.22	9.35 ± 0.05
<i>E. coli</i>	7.68 ± 1.12	7.27 ± 0.21	7.70 ± 0.29	7.23 ± 1.08	7.32 ± 0.47	7.41 ± 0.95
Coliforms	6.72 ± 0.96	6.48 ± 0.64	6.98 ± 0.44	6.33 ± 0.09	6.07 ± 0.56	6.37 ± 0.55
<i>Lactobacillus sp.</i>	7.86 ± 0.14	8.16 ± 0.56	8.44 ± 0.34	8.17 ± 0.38	8.35 ± 0.55	8.16 ± 0.67
<i>Clostridium perfringens</i>	4.63 ± 0.06	4.21 ± 0.62	3.55 ± 0.68	3.42 ± 0.91	3.15 ± 0.61	3.38 ± 0.89

C - Control group; E - Experimental group

¹CFU: colony forming unit

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

It was assumed that FB₁ induce immunosuppression in pigs or have the negative effects on the intestinal epithelial cell viability and proliferation (Bouhet and Oswald, 2007; Bracarense et al., 2012) leading the change of gastrointestinal microbial system. Lallès et al. (2009) proved a correlation between FB₁ consumption and the increase of stress protein in gastrointestinal track in pigs. Cytokine balance was altered after 1-week oral FB₁ feeding with 1.5 mg/kg bw and FB₁ decreased interleukin-4 (IL-4), increased interferon-gamma (IFN- γ) synthesis in the in vitro experiment (Taranu et al., 2005). Then Bouhet and coworkers (2006) reported that FB₁ (0.5 mg/kg bw for 7 days) has an effect on intestinal immune response by reducing the level of interleukin IL-8. However, the results from a few studies were controversial. 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 bw) could predispose in the colonization of pathogenic *E.coli* in pigs (Oswald et al., 2003; Devriendt et al., 2009) and with a dose of 11.8 μ g/kg, fumonisin transiently affects the balance of the digestive microbiota during the first four weeks of exposure. The change of microbiota was stronger in co-contamination with fumonisin and *Salmonella* (Burel et al., 2013). In this study, the growth of bacteria including *E. coli* in control groups was similar to experimental groups though there was the difference 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 also can be changed by different diets (Frese et al., 2015). The stability of the amount of caecal bacteria in this study showed that the gut microflora may be adapted themselves with the environmental

change. *Fusarium* can change the bacterial growth but only in short time while the effects of mycotoxin are usually in a long time and leading the chronic disease. In the future, longer time of experiment should be designed to achieve more information of the influence of *Fusarium* mycotoxin on the intestinal microorganisms.

Table 13: Number of bacteria in the pigs' caecal content with (experimental group) and without (control group) *Fusarium* measured by QPCR (log₁₀ copy number/g, means ± SD)

Bacteria	Period of the feeding time									
	Day0		Day2		Day4		Day6		Day8	
	C	E	C	E	C	E	C	E	C	E
Total bacteria	12.37 ± 0.18	12.46 ± 0.09	12.48 ^b ± 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> and <i>Prevotella</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
<i>Clostridium</i> <i>sp.</i>	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>Escherichia</i> <i>coli</i>	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>Enterobacteria</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
<i>Firmicutes</i>	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>Lactobacillus</i> <i>sp.</i>	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

C - Control group; E - Experimental group

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

7. Conclusions and recommendations

7.1. *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

The reduction of FB₁ concentration in chyme containing groups was sharper than it was in control 2 group. FB₁ concentration decreased while the HFB₁ increased. It is concluded that the caecal microflora of pigs can metabolise FB₁.

During the incubation period, the total number of cultured anaerobic bacteria declined while *Lactobacillus sp.* increased. Anaerobic bacteria such as *Lactobacillus sp.*, *Bacteroides* and *Prevotellat* tended to increase. Overall, FB₁ did not impact the growth of the investigated bacteria. Other kinds of bacteria should be investigated in similar experiments in the future. Additionally, the interaction between fumonisins and gut microbiota in *in vivo* experiments should be conducted as well.

7.2. *In vitro* effect of fumonisin B₁ on the ruminal microflora in sheep

Although the difference in the total bacteria number could not be observed, the amount of *Bacteroides* and *Prevotella* in the experimental group was higher than in the control group. That is why we speculate that the number of other bacterial types in the experimental group may have decreased and need further investigation. Other experiments should be carried out to clarify the relationship between FB₁ and *Bacteroides* and *Prevotella* according to the result of this study.

7.3. *In vivo* experiment: Effect of fumonisins producing *Fusarium sp.* on the microbiota in pigs

A change occurred in a short time regarding bacterial growth due to *Fusarium*, however, the effects of this mycotoxin are usually expressed after long exposure leading to chronic diseases. Therefore, a longer exposure period should be used in future experiments in order to get more information about the influence of FB₁ on the intestinal microbiota of pigs.

8. New scientific results

8.1. *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

No significant differences were observed between control 1 group (caecal content without FB₁) and experimental group (caecal content with FB₁). After 48 hour incubation, by culturing the number of aerobic bacteria, anaerobic bacteria, *E. coli*, Coliforms and *Lactobacillus* in the experimental groups were 7.26 ± 0.22 , 8.34 ± 0.08 , 6.16 ± 0.83 , 5.99 ± 0.86 and 8.01 ± 0.11 compared with 7.31 ± 0.19 , 8.39 ± 0.14 , 5.87 ± 0.66 , 5.84 ± 0.55 and 7.93 ± 0.12 (\log_{10} CFU/g) in the control 1 group, respectively while by qPCR, the number of total bacteria, *Lactobacillus*, *Bacteroides-Prevotella* were 11.79 ± 0.05 , 7.97 ± 0.11 and 11.33 ± 0.14 compared with 11.66 ± 0.13 , 7.83 ± 0.12 and 11.13 ± 0.15 (\log_{10} copy number/g), respectively.

8.2. *In vitro* effect of fumonisin B₁ on ruminal microbiota in sheep

No significant change was observed in total bacteria, *Firmicutes*, *Delta*- and *Gammaproteobacteria* while the *Bacteroides* and *Prevotella* group presented significant differences after 24 and 48-hour incubation, 8.36 ± 0.07 and 7.73 ± 0.04 compared with 8.48 ± 0.05 and 8.04 ± 0.16 (\log_{10} copy number/g), respectively.

8.3. *In vivo* experiment: Effect of fumonisins producing *Fusarium sp.* to the microbiota in pigs

This study achieved new results about the change of some bacteria in some points of feeding times. By plate count agar technique, the difference between control groups and experimental group was only presented in case of aerobic bacteria at Day_4, 8.60 ± 0.22 compared with 8.06 ± 0.20 (\log_{10}

CFU¹/g), respectively. Using the qPCR method, significantly different log₁₀ copy number/g were observed between the control and experimental group in total bacteria at Day_2 and Day_6, 12.48 ± 0.22 and 12.12 ± 0.28 compared to 12.11 ± 0.27 and 12.43 ± 0.21, respectively; in *Firmicutes* at Day_2, 10.52 ± 0.14 compared with 10.36 ± 0.10; in *E.coli* and *Enterobacteria* at Day_4, 9.65 ± 0.35 and 10.60 ± 0.39 compared with 8.97 ± 0.50 and 9.88 ± 0.38, respectively.

9. Summary

9.1. *In vitro* interaction between fumonisin B₁ and the intestinal microflora of pigs

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. Both classical (culturing) and modern (qPCR) microbiological methods were used for the determination of the changes of the selected bacterial types. The aerobic, anaerobic, coliforms, *Escherichia coli* and *Lactobacillus sp.* bacteria were cultured. Whereas the the total bacteria, *Lactobacillus*, *Bacteroides* and *Prevotella* species were investigated by the means of qPCR. No significant differences in the amount of bacteria groups between the experimental (buffer, chyme, and fumonisin B₁) and control 1 groups (buffer + chyme) were observed with both methods. FB₁ and hydrolysed FB₁ concentration were analysed by LC-MS. 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. FB₁ 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. HFB₁ was detected after 24 h incubation (0.012 ± 0 µg/ml). At 48 h incubation time, HFB₁ concentration was doubled to 0.024 ± 0.004 µg/ml. These results indicate that fumonisin B₁ can be metabolised by caecal microbiota in pigs although the number of studied bacteria were not altered.

9.2. *In vitro* effect of fumonisin B₁ on the ruminal microflora of sheep

The ruminal content of sheep was incubated anaerobically in McDougall buffer with and without fumonisin B₁ (FB₁) (5 µg/ml) for 0, 24 and 48 h. Two groups were designed including the experimental group (buffer, ruminal

content, FB₁) and the control group (buffer, ruminal content). The DNA copy number of the total bacteria, *Bacteroides* and *Prevotella*, *Firmicutes*, *Delta-* and *Gammaproteobacteria* were performed by quantitative polymerase chain reaction (qPCR) in the experimental and control group. The amount of *Bacteroides* and *Prevotella* in the experimental group was significantly higher ($P < 0.05$) than that of the control group while no differences were observed in the rest of the investigated bacterial species.

9.3. In vivo experiment: Effect of fumonisins producing *Fusarium sp.* to the microbiota in pigs

The fumonisins producing fungi, *Fusarium verticillioides*, was mixed in the diets of 7 piglets everyday in 9 days (FB₁ intake of 10 mg/animal) to investigate if there is any change of the caecal bacterial communities between the experimental groups (with *F. verticillioides*) and the control groups (without *F. verticillioides*). The plate count agar culturing technique was applied to measure amount of aerobic and anaerobic bacteria, *Escherichia coli*, *Coliform*, *Lactobacillus sp.* and *Clostridium perfringens*. The difference between the control groups and experimental group was only presented in the case of aerobic bacteria at Day₄, 8.60 ± 0.22 compared with 8.06 ± 0.20 ($P < 0.05$), respectively. Quantitative polymerase chain reaction was performed to estimate DNA copy number of total bacteria, *Bacteroides* and *Prevotella*, *Clostridium sp.*, *E.coli*, *Enterobacteria*, *Firmicutes* and *Lactobacillus sp.* The significant differences were observed between control and experimental group in total bacteria at Day₂ and Day₆, *Firmicutes* at Day₂, *E.coli* and *Enterobacteria* at Day₄. Regarding the entire feeding time, there was no considerable difference between both groups in all species of investigated bacteria by culturing technique and qPCR. Longer experiment time should be performed to gain more knowledge in the impact of *F. verticillioides* on the gastrointestinal bacteria.

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12. Publications related to the topic of dissertation

Articles:

1. Dang Huu Anh, Attila Zsolnai, Melinda Kovács, Nguyen Bá Hien (2016). Doc to nam moc fumonisin (2016). Khoa hoc ky thuat thu y / Veterinary sciences and techniques. Vol. 23, No. 7, 85-88.
2. Huu Anh Dang, Éva Varga-Visi, Attila Zsolnai (2016). Analysis of fumonisins: A review. Tap chi khoa hoc Nong nghiep Viet Nam / Vietnam Journal of Agricultural Sciences. Vol.14, No. 10, 1639-1649.
3. Huu Anh Dang, Attila Zsolnai, Melinda Kovács, István Bors, András Bónai, Bóta Brigitta, Judit Szabó-Fodor (2017). In vitro interaction between fumonisin B1 and the intestinal microflora of pigs. Polish Journal of Microbiology, Vol. 66, No. 2, 245-250. IF 0.746, Q3.
4. Huu Anh Dang, Attila Zsolnai, Melinda Kovács, Brigitta Bóta, Gábor Mihucz, Roland Pósa, Kinga Marosi, Mariam Kachlek, Judit Szabó-Fodor (2019). Effect of fumonisins producing *Fusarium verticillioides* on the microbiota in pig caecum. Acta Veterinaria Brno. In press: (2019) IF 0.422, Q3.

Conferences:

5. Huu Anh Dang (2015). Microbiological Methods in Control of Fumonisin Mycotoxins. In: Dávid Ágota , Dávid Ádám , Kardon Béla (szerk.). Excellent Science in ASEAN: Best Selected Papers and Posters from Young ASEAN Scientists on Water, Food and Health . 126 p. Konferencia helye, ideje: Budapest , Magyarország , 2015.03.17 -2015.03.19. Budapest: Regional Centre for Information and Scientific Development (RCISD), 2015. p. 124. (ISBN:978-963-12-1839-8).
6. Huu Anh Dang, Attila Zsolnai , Melinda Kovács , István Bors , András Bónai , Bóta Brigitta , Judit Szabó-Fodor (2016). In vitro interaction between fumonisin B₁ and the caecal microbiota of pigs. In: Dušan Kovačević (szerk.). VII International Scientific Agriculture Symposium "Agrosym 2016": Book of abstracts . 1226 p. Konferencia helye, ideje: Jahorina, Bosznia-Hercegovina, 2016.10.06-2016.10.09. Sarajevo: University of East Sarajevo, Faculty of Agriculture, 2016. p. 516. (ISBN:978-99976-632-6-9).

13. Publications not related to the topic of dissertation

1. Attila Zsolnai, Réka Szántó-Egész, Edit Ferencz-Elblinger, Anh Dang Huu, Anna Jánosi, Erika Koppányné Szabó, István Anton (2017). Loop-Mediated Isothermal Amplification based approach as an alternative to Recombinase Polymerase Amplification based detection of Mangalitza component in food products. Acta Alimentaria, Vol. 46, No. 3, 383-388. IF 0.357

14. Short professional CV

1. Personal details

Full name: Dang Huu Anh

Gender: Male

Date of birth: 1st September, 1982

Place of birth: Ha Long city, Quang Ninh province, Vietnam

Country of Citizenship: Vietnam

Passport number: B3991223

2. Contact Details

Address: Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Trau Quy town, Gia Lam district, Hanoi city, Vietnam

Private email address: bro.fvm.hua@gmail.com

3. Education Background

- **Qualifications (post-graduate):** Master of Agriculture

Name of institution: Hanoi University of Agriculture

Location of institution: Hanoi city, Gia Lam district, Trau Quy town

Start date: 20 October 2005; end date: 20 October 2007

Title of Masters Research graduate: *Procedure of vaccine manufacturing from duck enteritis virus strain DP-EG-2000*

- **Qualifications (undergraduate):** Doctor of Veterinary Medicine

Name of institution: Hanoi University of Agriculture

Location of institution: Hanoi city, Gia Lam district, Trau Quy town

Start date: 10 September 2000; end date: 20 August 2005

Title of DVM Research graduate: *Characterization of a virulent duck enteritis virus strain VG-2004*