# THESIS OF THE DOCTORAL (PhD) DISSERTATION

HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Institute of Physiology and Nutrition

Head of Doctoral School

PROF. DR. ANDRÁS SZABÓ, DSc

Supervisors

PROF. DR. MELINDA KOVÁCS

Member of the Hungarian Academy of Sciences (HAS)

DR. habil. VERONIKA HALAS

Associate Professor

## EVALUATION OF FUMONISINS EXPOSURE THROUGH STRUCTURAL AND FUNCTIONAL CHANGES IN THE GASTROINTESTINAL TRACT OF PIGS

Written by

YARSMIN YUNUS ZEEBONE

Kaposvár

2023

# 1. Research Background and Objectives

Considering the extreme weather events the world is now experiencing; it is crucial now more than ever to constantly monitor the occurrence and potential toxic pathways of mycotoxins since their prevalence is highly dependent on climatic conditions. Globally, aflatoxin $B_1$ ($AFB_1$), ochratoxin A (OTA), deoxynivalenol (DON), trichothecenes (TCTs), zearalenone (ZEN), and fumonisins (FUMs) are among the mycotoxins of toxicological concern.

The *Fusarium verticillioides* and *F. proliferatum* are the main plant pathogens that produce FUMs. Fumonisins (FUMs) commonly infect maize and maize- based products and result in diverse health implications when consumed. The well-known lethal clinical conditions of FUMs toxicity are the porcine pulmonary oedema (PPE) in pigs and equine leukoencephalomalacia (ELEM) in horses. Other species experience mainly liver and kidney injuries. The primary congeners of FUMs are fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$), fumonisin $B_3$ ($FB_3$) and fumonisin $B_4$ ($FB_4$). Among the types, $FB_1$ is the most toxic and the cellular pathway of $FB_1$ toxicity is its ability to disrupt sphingolipid metabolism through its resemblance to the enzyme ceramide synthase. This disruption results in disturbances in cellular pathways such as proliferation, apoptosis, and cell death. As such, the post-absorptive effects of FUMs are widely discussed leaving a significant gap in intestinal processes of gastrointestinal tract (GIT) functions.

The GIT performs several functions including digestion and absorption, immunity, barrier defense and maintaining the microbiota. Monogastric animal species like the pig have been reported to have relatively poor (1 - 6%) absorption of FUMs ingested and hence, the GIT is more exposed to the toxin than any other tissue. Further, in research, the effects of mycotoxins in younger animals are more detrimental than in older animals. The research therefore aimed to investigate the potentially toxic effects of dietary FUMs ($FB_1$ + $FB_2$ + $FB_3$) on the GIT's nutrients digestibility and mineral balance, digestive enzymes activity and intestinal morphology, antioxidant parameters, heat shock protein 70 (Hsp70) activity, serum biochemical parameters, histological and histopathological examinations of internal organs and, caecal fermentation products as well as intestinal and faecal microbiota composition of nursery pigs in a 3- week- long feeding trial.

A further objective was to ascertain the potential effects that a higher dose of FUMs (40 mg/kg diet) on the amino acids would have on the digestibility in fattening pigs.

# 2. Materials and Methods

## 2.1. Nursery pigs' study

### 2.1.1. Animals, feeding and housing

The experiment was carried out according to the regulations of the Hungarian Animal Protection Act. The allowance number for the studies was SOI/31/00308-10/2017 (date of approval: 28 February 2017, by the Hungarian National Scientific Ethical Committee on Animal Experimentation and issued on 27 March 2017 by the Somogy County Government Office, Department of Food Chain Safety and Animal Health).

A total of 18 male weaned Danbred pigs at five weeks old with weights that averaged 13.5 ± 1.3 kg were included in the study. The pigs were given a 2- week physiological acclimatization period and at exactly 7 weeks of age, were enrolled in the feeding trial. A group was allotted to a diet that contained no fungal culture of FUMs [($FB_1$ + $FB_2$ + $FB_3$); control group (G1)], another group consumed a diet containing 15 mg/kg FUMs (G2) and the third group consumed a 30 mg/kg FUMs (G3) diet (n = 6 / group) for 21 days. Titanium dioxide ($TiO_2$; 0.5%) was added to the feed as an indigestible marker to evaluate apparent total tract digestibility (ATTD). Feed was of commercial origin (maize and soybean meal- based diet), offered as an amount that covers 2.5 times the maintenance energy requirement, and was provided twice a day in two equal portions. Leftover feed was measured back every day and drinking water was made available *ad libitum*.

Pigs were kept in individual metabolic cages (80 x 80 cm) located in the Experimental Animal Unit of the Department of Farm Animal Nutrition at the Hungarian University of Agriculture and Life Sciences, Kaposvár Campus (MATE-KC). Pigs were weighed individually with gram precision at the beginning of the trial and at weekly intervals and, their health status was monitored every day. The room temperature was adjusted according to the needs of the nursery pigs and the diarrhoea score was monitored throughout the trial.

Preparation of FUMs was *in vitro* according to Fodor *et al*. (2006) and was mixed into the rations of the pigs to contain 15.40 and 29.75 mg FUMs/ kg diet for the two contaminated diets. Diet fed to the control group did not contain detectable amounts of FUMs as well as DON, ZEN and T-2 toxin which was tested and confirmed.

At the end of the trial period, pigs were euthanized by exsanguination after sedation (Euthanyl-Pentobarbital Sodium, 400 mg/mL, Dechra Veterinary Products, Shrewsbury, UK). Blood samples were taken and, internal organs including the liver, kidney, lungs, pancreas, small intestinal contents and sections were sampled, weighed, and stored appropriately for further analysis.

2.1.2. Metabolic study

From day 17 to 21 of the trial, faecal and urine samples were collected separately, weighed, and immediately frozen at -18 °C. Approximately 200 g of faeces were collected 4 times a day. For the balance study, urine samples from each day were combined and strained through glass wool to remove any particulate matter or debris. To effectively trap ammonia, urine was collected over sulphuric acid and maintained at a pH of 2 or lower.

All laboratory analyses were conducted at the Chemistry Laboratory of MATE-KC. According to methods described in the Association of Official Analytical Chemists (AOAC, 2000), the chemical compositions of the diets and faecal samples were analysed and used to calculate the ATTD of crude protein (CP), ether extract (EE), crude fibre (CF), starch, ash, calcium (Ca), phosphorus (P) and the dietary minerals [i.e., magnesium (Mg), potassium (K), sodium (Na), copper (Cu), and zinc (Zn)]. The $TiO_2$ was analyzed by the lab's own analytical procedure.

The gross energy (GE) content of the feeds, faecal, and urine samples was determined by analyzing duplicate samples using IKA-Calorimeter C4000 adiabatic bomb calorimeter with benzoic acid used as a standard. For the urinary energy, a total of 16.0 g of filtered subsample urine was added to 4.0 g of dried cellulose and subsequently freeze-dried for 24 h. The energy content of cellulose was also determined, and urinary energy was calculated by subtracting the energy in cellulose from the energy in the samples containing both urine and cellulose.

- The ATTD (g/g) of the nutrients CP, EE, CF, starch, and ash, as well as the minerals, Ca, P, Mg, K, Na, Cu, and Zn was calculated using the $TiO_2$ concentration in faeces and feed and calculated in DM basis with the equation:

$$ATTD, g/g = 100 - (100 * \frac{Marker\ in\ feed * Nutrient\ or\ mineral\ in\ feces}{Marker\ in\ feces * Nutrient\ or\ mineral\ in\ feed})$$

- The retention of nitrogen (N), P, Ca, Mg, Cu, Zn, K, and Na was calculated using the equation:

$$MR = Mi - Mof - Mou$$

where *MR* is the mineral retention (g/ 5d), *Mi* is dietary mineral intake (g/ 5d), *Mof* (g or mg/ 5d) is mineral output via faeces (g or mg/ 5d) and *Mou* (g or mg/ 5d) is mineral output via urine (g), respectively. The faecal mineral output was calculated from the feed intake, the mineral content of the feed and the digestibility rate, while urine mineral output was calculated from the amount of urine excreted multiplied by the mineral content in the urine.

- The dietary digestible energy (DE) content was calculated using the equation:

$$DE\ (MJ/kg) = \ Gross\ Energy\ (MJ/kg) * Energy\ Digestibility\ coefficient$$

- The dietary metabolizable energy (ME) content was calculated using the equation:

$$ME = (DEi - Eu)/FI$$

where ME is the dietary metabolizable energy (MJ/kg), DE is digestible energy intake (MJ/ 5d), Eu is the energy excreted via urine (MJ/ 5d) and FI is the feed intake (kg/ 5d), respectively.

2.1.3. Determination of gastrointestinal tract function

The histology of the small intestinal segments was done by collecting approximately 3 cm long segments of the small intestine from the duodenum (approximately 6 cm distal from the pylorus), jejunum (from the middle part) and ileum (approximately 6 cm proximal to the ileocecal junction). Formalin-fixed (10% buffered formalin; pH 7.2) samples were embedded in paraffin and sectioned in transverse. After deparaffinization and dehydration, tissues were processed by standard histologic techniques to produce 4 μm thick sections which were then stained with hematoxylin and eosin (H.E.). The structure of the mucosa was observed using an ocular micrometer to examine the orientation of the villus height and crypt depth. Measurement of villi height (VH) and crypt depth (CD) was done by taking cross-sections of 5 randomly selected villi. The ratio of the villus to the crypt was estimated by dividing the villus height by the crypt depth (Autopsy Company, Budapest, Hungary).

Contents of the small intestine and pancreas to examine digestive enzyme activity were diluted with a phosphate buffer (pH 7). Lactase and maltase activities were determined in the duodenum, jejunum, and ileum of the small intestine. Amylase, protease, and lipase activities were determined

in the pancreas. Dry matter was determined by heating at 105 °C for 24 h. These measurements were performed in cooperation with the Department of Microbiology, Nutrition and Dietetics, Czech University of Life Sciences, Prague (Czech Republic).

About 3 g of caecal digesta was homogenized with 4.5 ml metaphosphoric acid (4.16%), then centrifuged at 10,000 g for 10 min and filtrated. The concentration of SCFAs was measured with gas chromatography (Shimadzu GC 2010, Japan. A Nukol 30 m x 0.25 mm x 0.25 μm capillar column (Supelco, Bellefonte, PA, USA) was used for separation and FI detector. The split ratio was 1:50, 1 μl was the injected volume, carrier gas was helium at 0.84 ml/min. Detector conditions: air 400 ml/min, hydrogen 47 ml/min, temperature: injector 250 °C, detector 250 °C, column 150 °C). 2-etil-butyrate (FLUKA Chemie GmbH, Buchs, Switzerland) was used as internal standard.

For the small intestinal microbiota analysis, samples were taken from the duodenum, jejunum, and ileum. Samples were then weighed and stored appropriately for further analysis. Amplicon sequencing of the V3-V4 region of the 16S rRNA gene was performed by LGC Genomics GmbH (Berlin, Germany). Library preparation and sequencing were performed using an Illumina MiSeq platform with v3 chemistry. DNA fragments were amplified using amplification primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013). Primers also contained the Illumina sequencing adapter sequence and a unique barcode index. Resulting amplicons were sequenced using the Illumina MiSeq v3 600-cycle kit to provide paired-end read lengths of 2 x 300 bp. Demultiplexing of all libraries for each sequencing lane were attained using the Illumina bcl2fastq 2.17.1.14 software (Illumina Inc., San Diego, USA). Combination of forward and reverse reads were carried out using the BBMerge 34.48 tool (Bushnell *et al*., 2017). The mothur software package [(v1.35.1, (Schloss *et al*., 2009)] was used for pre-processing and operational taxonomic units (OTUs) picking from Illumina amplicon sequencing data by clustering at the 97 % identity level. Creation of relative abundance taxonomical tables in an Excel format for was performed with QIIME 1.9.0. (LGC Genomics GmbH, Berlin, Germany)(Caporaso *et al.*, 2010).

### 2.1.4. Determination of general health effects

Fresh blood samples were taken during exsanguination into heparin tubes (20 IU/mL whole blood). Samples were centrifuged for 10 min at 1000 × g (SIGMA 3-30KS refrigerated centrifuge,

Osterode am Harz, Germany) for plasma separation. The concentration of plasma total protein (TP), albumin (ALB), creatinine concentrations, the activities of the alanine aminotransferases (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), lactate dehydrogenase and alkaline phosphatase (ALKP) were determined using Roche Hitachi 912 Chemistry Analyzer (Hitachi, Tokyo, Japan) and commercial diagnostic reagent kits (Diagnosticum Ltd., Budapest, Hungary) at the Vet-Med Laboratory, Budapest (Hungary).

For the determination of lipid peroxidation and antioxidant status, samples were stored at -80 °C until analysis. Lipid peroxidation was determined by the quantification of malondialdehyde (MDA) level with the 2-thiobarbituric acid method in cell hemolysate (Botsoglou *et al*., 1994). The concentration of reduced glutathione (GSH) was measured by Sedlak and Lindsay (1968) and the activity of glutathione peroxidase (GSHPx) according to Lawrence and Burk (1978).

Following macroscopic external and internal examination of the organs, the liver, kidney, lung, and small intestine were fixed in 10% buffered formaldehyde and embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin-eosin and examined by light microscopy.

#### 2.1.5. Statistical analysis

The statistical analyses of the digestibility and mineral retention, intestinal morphology, enzyme activity, antioxidant parameters, Hsp70 activity, SCFAs and serum biochemical measurements data were performed using the SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Results were subjected to a one-way analysis of variance (ANOVA). In case of a significant treatment effect, the intergroup differences were checked by a Tukey post-hoc test. A $P$ value < 0.05 was described as significant (SPSS, 2012).

For the microbiota statistical analysis, creation of relative abundance taxonomical tables in an Excel format for was performed with QIIME 1.9.0. (LGC Genomics GmbH, Berlin, Germany) (Caporaso *et al.*, 2010). Relative abundance data generally did not follow a normal distribution, as assessed by the Shapiro-Wilk test (Ju and Zhang, 2015). Differential abundance testing in the duodenum, jejunum, and ileum content, and faeces of the three treatment groups [Control (G1), 15 mg/kg FUMs (G2) and 30 mg/kg FUMs (G3)] was performed by the non-parametric Kruskal-Wallis test using IBM SPSS Statistics 27.0 software (SPSS Inc., Chicago, IL, USA (Hazra and Gogtay, 2016; Weiss *et al.*, 2017; Xia and Sun, 2017). A difference was considered significant at $P < 0.05$. The bacterial composition of day 0 and day 21 faecal samples in the three treatment

groups was compared using the Wilcoxon Signed-Rank test (Ju and Zhang, 2015; Hazra and Gogtay, 2016; Chen *et al*., 2017).

### 2.2. Cannulated fattening pigs' study

The experiment was carried out according to the regulations of the Hungarian Animal Protection Act. The allowance number for the studies was SOI/31/00997-7/2018.

A total of ten Danbred breed fattening pigs (average bodyweight: 65.5 ± 4.1 kg) fitted with a post-valve T- cecum (PVTC) cannula was enrolled in the study. Pigs were kept in individual crates (200 x 120 cm) located in the Experimental Animal Unit of the Department of Animal Nutrition (MATE-KC). A maize-soybean-based diet of commercial origin was used as the basal diet. The pigs were then randomly assigned to 2 different diets; a control diet that contained no FUMs ($FB_1$ + $FB_2$ + $FB_3$) nor any trace of other mycotoxins (tested and confirmed) and a 40 mg/kg FUMs-contaminated diet (n = 5 animals/group). The study had a 2 x 2 factorial design, the independent variables were the treatments (control or 40 mg/kg intoxication) and the exposure time [7 days (short- exposure) or 21 days (long exposure)]. The pigs were weighed individually at the beginning of the trial and the end of the trial and, their health status was monitored every day. The room temperature was adjusted according to the breeder's guidelines.

Finely ground fungal culture produced according to Fodor *et al*. (2006) containing FUMs ($FB_1$ + $FB_2$ + $FB_3$) was mixed carefully and thoroughly into the ration of the experimental animals, to provide 40 mg/kg FUMs contaminated diet. Diet fed to the control group did not contain detectable amounts of FUMs as well as DON, ZEN, and T-2 toxin. Feeding time was twice daily (at 07:30 h and 15:30 h) and in equal proportions. Ileal digesta samples were collected (08:00 h to 20:00 h) in durable plastic bags around the PVTC cannula secured with rubber bands. After day 6 of feeding experimental diets, and for 3 consecutive days from then on i.e., on days 7, 8, and 9, the first collection period begun and was labelled as the short-term exposure period. After day 20 of feeding experimental diets and for 3 consecutive days as well, i.e., on days 21, 22, and 23, the second collection period began and was labelled the long-term exposure period. The collected ileal digesta samples were weighed and immediately stored at -20 °C to prevent bacterial degradation of the amino acids.

Chemical analysis of crude protein (CP) and the AAs – aspartic, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine,

histidine, lysine, ammonia, and arginine was measured using MSZ EN ISO 13903:2005 standardization procedure. Nitrogen was determined with the Kjeldahl method and the $TiO_2$ was also analyzed.

Apparent ileal digestibility (AID) of the CP and AAs was calculated using the $TiO_2$ concentration in ileal digesta samples and feed with the equation:

$$AID, g/g = \left[1 - \left(\frac{\%\, TiO_2\ in\ feed * \ \%\, CP\ or\ AA\ in\ ileal\ digesta}{\%\, TiO_2\ in\ ileal\ digesta * \ \%\, CP\ or\ AA\ in\ feed}\right)\right]$$

Statistical analyses were performed using the SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Results were subjected to a two-way ANOVA of the SPSS version 20.0 software using FUM dose (i= 2; 0 or 40 mg FUMs/ kg feed) and duration (j= 2; short vs long-term exposure) as fixed factors. A *P* value of < 0.05 was considered significant (SPSS, 2012).

## 3. Results

Given the present dosages of dietary FUMs used (15 or 30 mg/kg diet) for the nursery pigs' trial or 40 mg/kg for the fattening pigs' trial, no mortality nor diseases were observed during both trials. Transitional diarrheic episodes were seen in the nursery pigs during the 2-week acclimatization period. However, this was completely alleviated before the intoxication period began.

### 3.1. Dietary fumonisins impair nutrient digestibility and mineral balance without hampering digestive enzyme activity or intestinal architecture in nursery pigs

Dietary FUMs at a dose of 15 (G2) or 30 (G3) mg/kg significantly lowered the apparent total tract digestibility (ATTD) of CF, ash, Ca and P as compared to the control group (G1) (Table 1).

**Table 1.** Effect of varied levels of dietary fumonisins on apparent total tract digestibility (%) of nutrients in nursery pigs (data are means ± standard deviation (SD) of 6 individuals/group)

| ATTD, % | Control (G1) | 15 mg/kg FUMs (G2) | 30 mg/kg FUMs (G3) |
|---|---|---|---|
| Dry matter | 84.8 ± 1.0 | 83.6 ± 0.5 | 83.8 ± 1.1 |
| Crude protein | 83.8 ± 2.1 | 83.3 ± 1.4 | 82.3 ± 1.2 |
| Ether extract | 64.3 ± 7.3 | 56.2 ± 8.6 | 57.1 ± 9.6 |
| Crude fiber | 44.8 ± 1.4 **b** | 40.5 ± 2.5 **a** | 40.6 ± 3.0 **a** |
| Starch | 99.1 ± 0.2 | 98.9 ± 0.1 | 99.1 ± 0.2 |
| Ash | 58.6 ± 3.3 **b** | 52.6 ± 1.7 **a** | 53.6 ± 1.6 **a** |
| Calcium | 65.4 ± 6.6 **b** | 56.3 ± 3.4 **a** | 53.9 ± 6.9 **a** |
| Phosphorus | 67.5 ± 3.5 **b** | 62.6 ± 1.5 **a** | 60.4 ± 3.6 **a** |

ATTD= apparent total tract digestibility. Different lowercase letter indexes, a or b, without common letter in the same row differ significantly at $P < 0.05$

The retention study revealed a much higher Ca excretion via faeces in G3 while the opposite was observed for excretion via the urine. Phosphorus excretion through faeces was notably high in G2 and G3 as compared to G1. However, through urine, P excretion was notably high in G1 as compared to G2 or G3. The ATTD and relative retention rates of N, K, Na and Mg were not affected by dietary treatments. The microminerals – Zn and Cu analyzed in the study had reduced ATTD rates and further exhibited poor relative retention in G2 and G3 compared to G1 (Table 2).

**Table 2.** Effect of varied levels of dietary fumonisins on retention (mg/ 5d) of calcium, phosphorus, zinc, and copper in nursery pigs (data are means ± standard deviation (SD) of 6 individuals/group)

| Groups | Control (G1) | 15 mg/kg FUMs (G2) | 30 mg/kg FUMs (G3) |
|---|---|---|---|
| ATTD (%) of DM | 84.8 ± 1.0 | 83.6 ± 0.5 | 83.8 ± 1.1 |
| *Calcium, g/ 5d* | | | |
| Intake | 23.9 ± 0.0 | 23.9 ± 0.0 | 23.7 ± 0.4 |
| ATTD (%) | 65.3 ± 6.6 **b** | 56.3 ± 3.4 **a** | 54.0 ± 9.4 **a** |
| Output in faeces | 8.3 ±1.6 **a** | 10.5 ± 0.8 **ab** | 11.6 ± 2.0 **b** |
| Output in urine | 2.8 ±1.7 **b** | 1.4 ± 0.7 **ab** | 1.1 ± 0.5 **a** |
| Retention | 12.8 ± 2.6 | 12.1 ± 0.7 | 11.7 ±1.4 |
| Retained: intake | 0.54 ± 0.1 | 0.51 ± 0.0 | 0.49 ± 0.1 |
| *Phosphorus, g/ 5d* | | | |
| Intake | 20.28 ± 0.0 | 20.28 ± 0.0 | 20.13 ± 0.4 |
| ATTD (%) | 67.5 ± 3.5 **b** | 62.6 ± 1.5 **a** | 60.0 ± 3.6 **a** |
| Output in faeces | 6.6 ± 0.7 **a** | 7.6 ± 0.3 **b** | 7.9 ± 0.6 **b** |
| Output in urine | 1.4 ± 0.8 **b** | 0.6 ± 0.2 **a** | 1.1 ± 0.4 **ab** |
| Retention | 12.3 ±1.2 | 12.2 ± 0.5 | 11.1 ± 0.9 |
| Retained: intake | 0.61 ± 0.1 | 0.60 ± 0.0 | 0.55 ± 0.0 |
| *Zinc, mg/ 5d* | | | |
| Intake | 737.1 ± 0.0 | 737.1 ± 0.0 | 731.7 ± 0.01 |
| ATTD (%) | 20.6 ± 1.7 **b** | - 13.7 ± 7.7 **a** | - 15.4 ± 8.3 **a** |
| Output in faeces | 585.2 ± 86.4 **a** | 837.9 ± 56.6 **b** | 844.9± 72.5 **b** |
| Output in urine | 15.1 ± 3.2 | 16.7 ± 8.5 | 12.9 ± 8.0 |
| Retention | 136.8 ± 88.7 **b** | - 117.6 ± 51.4 **a** | - 126.1 ± 61.3 **a** |
| Retained: intake | 0.19 ± 0.1 **b** | - 0.16 ± 0.1 **a** | - 0.17± 0.1 **a** |
| *Copper, mg/ 5d* | | | |
| Intake | 78.0 ± 0.0 | 78.0 ± 0.0 | 77.4 ± 0.0 |
| ATTD (%) | 20.3 ± 6.3 **b** | 5.8 ± 3.9 **a** | 11.9 ± 3.9 **a** |
| Output in faeces | 62.1 ± 4.9 | 97.2 ± 58.1 | 68.2 ± 3.5 |
| Output in urine | 0.7 ± 2.7 | 0.7 ± 2.7 | 0.9 ± 2.1 |
| Retention | 15.2 ± 4.9 **b** | 3.9 ± 2.8 **a** | 8.4 ± 3.2 **a** |
| Retained: intake | 0.19 ± 0.1 **b** | 0.05 ± 0.0 **a** | 0.11 ± 0.0 **a** |

ATTD= apparent total tract digestibility, DM= dry matter. Different lowercase letter indexes, a or b, without common letter in the same row differ significantly at $P < 0.05$

The GE intake, energy excreted via faeces, energy excreted via urine, and ME content of the feed remained unaffected by dietary FUMs. Meanwhile, the DE was lower in G2 (13.85 MJ/kg) or G3 (13.93 MJ/kg) as compared to G1 (14.1 MJ/kg).

Feeding either 15 or 30 mg/kg FUMs diet had no impact on the activities of pancreatic or brush border enzymes of the nursery pigs following dietary exposure of 21 days compared to the non-contaminated diet-fed counterparts. Neither was there any notable effect on the small intestinal architecture. The caecal fermentation outputs (formic acid, acetic acid, propionic acid, valeric acid) analyzed also remained unaffected by treatments.

From the genus level perspective of the intestinal and faecal microbiota analysis, a total of 13 significant differences were detected. These were a notable reduction in the abundance of *Alloprevotella*, *Campylobacter* and *Incertae Sedis* in the duodenum; *Turicibacter* in the jejunum; and *Clostridium sensu stricto I*. in the ileum. Higher levels of *Solobacterium, Faecalibacterium, Anaerofilum*, *Ruminococcus*, *Subdoligranulum*, *Pseudobutyrivibrio*, *Coprococcus* and *Roseburia* were observed in the faecal microbiota (Table 3).

**Table 3.** Colonization pattern observed in animals (6 pigs/ group) fed either a control diet (G1), and 15 or 30 mg/kg FUMs (G2 and G3, respectively) contaminated diets for 21 days as compared to the control group (G1)

| Sample type | Significant changes in relative abundance | Group effect | *P*-value |
|---|---|---|---|
| Duodenum | Bacteroidales: Prevotellaceae: *Alloprevotella* | G1G3 ↓ | 0.013 |
| Duodenum | Campylobacteraceae: *Campylobacter* | G1G3, G2G3 ↓ | 0.016, 0.012 |
| Duodenum | Firmicutes: Clostridiales: Lachnospiraceae: *Incertae Sedis* | G1G3, G2G3 ↓ | 0.009, 0.035 |
| Jejunum | Firmicutes: Erysipelotrichaceae: *Turicibacter* | G1G3 ↓ | 0.001 |
| Ileum | Clostridiales: Clostridiaceae: *Clostridium sensu stricto 1* | G1G3 ↓ | 0.009 |
| Faeces | Firmicutes: Erysipelotrichaceae: *Solobacterium* | G1G3, G2G3 ↑ | 0.040, 0.003 |
| Faeces | Firmicutes: Clostridiales: Ruminococcaceae: *Faecalibacterium* | G2G3 ↑ | 0.020 |
| Faeces | Firmicutes: Clostridiales: Ruminococcaceae: *Anaerofilum* | G1G3, G2G3 ↑ | 0.027, 0.008 |
| Faeces | Firmicutes: Clostridiales: Ruminococcaceae: *Ruminococcus* | G2G3 ↑ | 0.011 |
| Faeces | Firmicutes: Clostridiales: Ruminococcaceae: *Subdoligranulum* | G2G3 ↑ | 0.015 |
| Faeces | Firmicutes: Clostridiales: Lachnospiraceae: *Pseudobutyrivibrio* | G1G3, G2G3 ↑ | 0.031, 0.027 |
| Faeces | Firmicutes: Clostridiales: Lachnospiraceae: *Coprococcus* | G2G3 ↑ | 0.04 |
| Faeces | Firmicutes: Clostridiales: Lachnospiraceae: *Roseburia* | G2G3 ↑ | 0.011 |

Symbols are the following= ↑ indicates an increase, ↓ indicates a decrease between the groups, G1= control group, G2= 15 mg/kg FUMs dosed group, G3= 30 mg/kg FUMs dosed group

Although not statistically different, *Lactobacillus sp*. present in the duodenum, ileum and jejunum showed a typical dose-response increment trend in the order of G1–G2–G3. The same trend could be identified in the faeces but was extremely low.

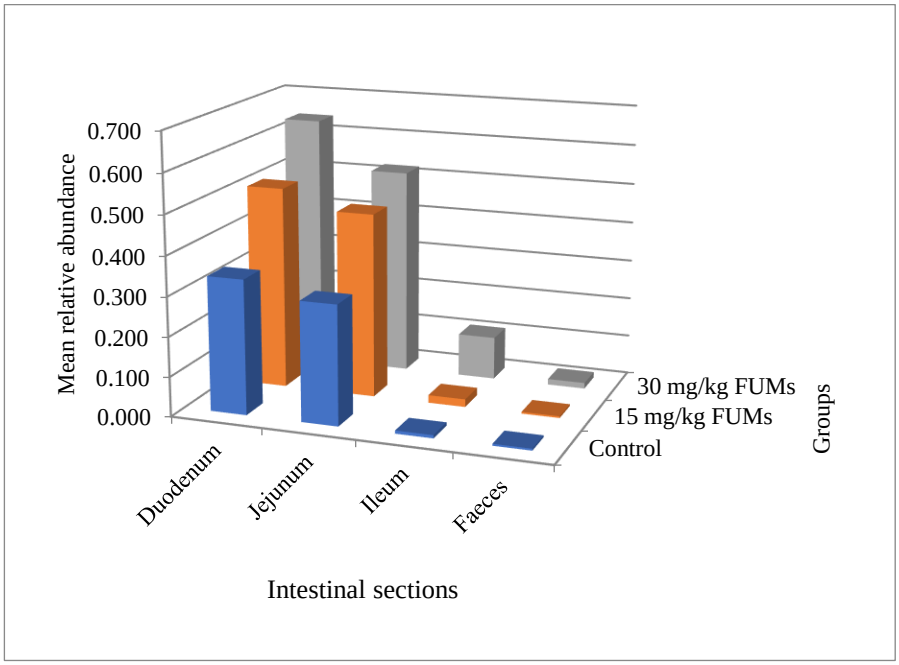


**Figure 1**. Mean relative abundance of *Lactobacillus* in the intestinal and faecal microbiota of nursery pigs

### 3.2. Effects of dietary fumonisins on modulating some health indicators in nursery pigs

The blood total protein concentration was significantly increased ($P = 0.01$) in G3 as opposed to G1 and G2. AST, GGT and ALKP activities were all significantly elevated ($P < 0.05$) in G3 compared to G1; G2 was indifferent regarding GGT and ALKP levels. CK was significantly elevated ($P = 0.02$) to about 3 folds in G3 compared to G1 and G2. There was also a notable rise ($P = 0.003$) in LDH activity in G3 compared to G1 or G2. Feeding pigs with a 30 mg/kg FUMs-contaminated diet significantly elevated ($P < 0.05$) creatinine and cholesterol concentrations as well as Ca and Mg levels compared to G1 or G2. When compared to the other two groups, G3 had a significantly declined GFR ($P = 0.03$). However, there was no dietary FUMs influence ($P > 0.05$) on the serum levels of albumin, ALT, urea, Fe, Na and Cl in all groups. Meanwhile, none of the antioxidant parameters, i.e., GSH and GSHPx and MDA as the end-product of lipid peroxidation determined in the lung, liver, kidney, or plasma exhibited a significant alteration in their activity or levels of pigs regardless of treatment.

Histopathological alterations of the liver and kidney revealed FUMs effects in both exposed groups compared to the control. Liver cells of animals receiving FUMs diets exhibited decreased glycogen content. In the kidneys, tubular epithelial detachment and, in this context, clusters of collapsed epithelial cells detached in the cavity of the drainage ducts of the marrow, as well as scattered

inter-tubular foci of lymphohistiocytic infiltrations were observed in all animals in G3 and not G1 or G2. Furthermore, in both intoxicated groups, mild focal lymphohistiocytic infiltrates were seen interstitially, and mild focal fibrotic thickening of the pleural covering the lungs was observed. No signs of severe pulmonary oedema were observed, however.

### 3.3. Effect of dietary fumonisin on the apparent ileal digestibility of crude protein and amino acids in fattening pigs

The main effect of duration (d), and the interaction of treatment (t) and duration (t x d) tended to impact the AID of histidine ($P$ = 0.001 and 0.047, respectively). On the other hand, only t x d effect was observed for arginine and tyrosine (($P$ = 0.003 and 0.047, respectively) (Table 4).

**Table 4**. Effect of long or short-term exposure of dietary fumonisins on apparent ileal digestibility (AID) of dry matter, crude protein, and amino acids (AAs) in fattening pigs (data are means ± standard deviation (SD) of 5 individuals/group)

| Duration, d | Short-term effect, 7 days | | Long-term effect, 21 days | | *P* -values | | |
|---|---|---|---|---|---|---|---|
| Treatments, t | Control | 40 mg/kg FUMs | Control | 40 mg/kg FUMs | Treatment (t) effect | Duration (d) effect | Interaction (t x d) |
| *AID of AAs, g/g* | | | | | | | |
| Dry matter | 0.7404 ± 0.013 | 0.7467 ± 0.011 | 0.7641 ± 0.027 | 0.7432 ± 0.007 | 0.29 | 0.16 | 0.06 |
| Crude protein | 0.7737 ± 0.011 | 0.7927 ± 0.017 | 0.7876 ± 0.007 | 0.7778 ± 0.022 | 0.58 | 0.95 | 0.099 |
| *Amino acids* | | | | | | | |
| **Arginine** | 0.8524 ± 0.007 | 0.8659 ± 0.005 | 0.8642 ± 0.009 | 0.8500 ± 0.007 | 0.93 | 0.58 | **0.003** |
| Threonine | 0.7032 ± 0.024 | 0.7094 ± 0.024 | 0.7082 ± 0.026 | 0.6866 ± 0.011 | 0.494 | 0.43 | 0.23 |
| Valine | 0.7668 ± 0.020 | 0.7734 ± 0.019 | 0.7614 ± 0.015 | 0.7553 ± 0.017 | 0.98 | 0.23 | 0.50 |
| Phenylalanine | 0.8031 ± 0.018 | 0.8149 ± 0.016 | 0.8128 ± 0.007 | 0.8048 ± 0.014 | 0.803 | 0.98 | 0.22 |
| Methionine | 0.8743 ± 0.020 | 0.8867 ± 0.013 | 0.8780 ± 0.005 | 0.8708 ± 0.012 | 0.71 | 0.39 | 0.18 |
| Lysine | 0.8394 ± 0.009 | 0.8426 ± 0.011 | 0.8593 ± 0.15 | 0.8508 ± 0.013 | 0.70 | 0.06 | 0.41 |
| **Histidine** | 0.7886 ± 0.020 | 0.8084 ± 0.015 | 0.8458 ± 0.008 | 0.8325 ± 0.009 | 0.67 | < **0.001** | **0.047** |
| Isoleucine | 0.7934 ± 0.020 | 0.8062 ± 0.021 | 0.7973 ± 0.009 | 0.7945 ± 0.019 | 0.60 | 0.68 | 0.42 |
| Leucine | 0.8307 ± 0.024 | 0.8431 ± 0.019 | 0.8416 ± 0.008 | 0.8341 ± 0.018 | 0.80 | 0.92 | 0.31 |
| **Tyrosine** | 0.6958 ± 0.030 | 0.7130 ± 0.013 | 0.7260 ± 0.004 | 0.7075 ± 0.010 | 0.94 | 0.15 | **0.047** |
| Alanine | 0.7661 ± 0.016 | 0.7725 ± 0.015 | 0.7471 ± 0.018 | 0.7506 ± 0.25 | 0.63 | 0.07 | 0.89 |
| Glutamic acid | 0.8421 ± 0.013 | 0.8546 ± 0.006 | 0.8496 ± 0.008 | 0.8393 ± 0.015 | 0.86 | 0.53 | 0.08 |
| Glycine | 0.5863± 0.034 | 0.6169 ± 0.057 | 0.6308 ± 0.008 | 0.6070 ± 0.051 | 0.88 | 0.46 | 0.25 |
| Cysteine | 0.7184 ± 0.021 | 0.7368 ± 0.027 | 0.7559 ± 0.011 | 0.7342 ± 0.019 | 0.88 | 0.12 | 0.08 |
| Aspartic acid | 0.7708 ± 0.015 | 0.7771 ± 0.010 | 0.7782 ± 0.011 | 0.7686 ± 0.010 | 0.79 | 0.93 | 0.28 |
| Proline | 0.6874 ± 0.069 | 0.6811 ± 0.116 | 0.5993 ± 0.103 | 0.6891 ± 0.040 | 0.400 | 0.418 | 0.34 |
| Serine | 0.7724 ± 0.016 | 0.7802 ± 0.016 | 0.7906 ± 0.010 | 0.7760 ± 0.016 | 0.671 | 0.39 | 0.18 |

# 4. Conclusions

1. The present study revealed the consequence of dietary fumonisins with regards to digestion of nutrients in the gastrointestinal tract. A dietary concentration of 15 or 30 mg/kg FUMs had adverse effects on nutrient digestibility and microminerals retention without affecting overall growth performance of nursery pigs following 21 days of exposure. This implies a decreased nutritional value of the mixed feed.
2. Fumonisins' peculiar effect on liver and kidney was attested in the present research. Hepatotoxic and nephrotoxic state as marked by the perturbations in serum AST, LDH, ALT, CK, and total cholesterol were observed in the study. Also, some histopathological alterations were seen in the liver and kidney which are indicative of organ injury induced by dietary fumonisins.
3. A high expression of Hsp70 activity in the liver of the group receiving the largest fumonisins could be interpreted as a sign of a possible counteraction to a potential induction of oxidative damage, even if none of the oxidative damage biomarkers demonstrated any effect.
4. The presence of dietary fumonisins in concentrations of 15 or 30 mg/kg diet did not affect the short -chain fatty acids concentrations. However, the faecal populations were shifted to the abundance of gut-health-promoting families such as Ruminococcaceae and Lachnospiraceae and their respective genera *Solobacterium, Faecalibacterium, Anaerofilum, Ruminococcus, Subdoligranulum, Pseudobutyrivibrio, Coprococcus*, and *Roseburia.* And although *Lactobacillus* was statistically unremarkable, the observed abundance trends present in the duodenum, ileum, and jejunum displayed a typical dose-response increment pattern, with the highest abundance in the duodenum and a decreasing trend in the distal direction of the intestine.
5. Following their exposure to a high concentration of 40 mg/kg FUMs for 7 or 21 days to assess the impact on crude protein and amino acids digestibility, the resulting effect of less impact revealed by this dosage implies the resilience of fattening pigs at such a growth stage and thus, their ability to manage dietary fumonisins better.

# 5. Recommendations

The current research looked at the impact of dietary fumonisins on pigs' structural and functional integrity of the gastrointestinal tract and microbiota. Some results such as the noticeable changes in nutritional digestibility, some health markers, and a putative shift in the microbial community were expected. The results on trace mineral metabolism in the nursery pigs were however vague and could be treated as a preliminary outcome. The following recommendations are thus, advised:

1. To better acknowledge the impact of fumonisins on mineral metabolism following our findings, a further trial should be carried out to ascertain the exact toxic mechanism induced by fumonisins in impeding absorptive processes in the GIT. Perhaps, a change of methodology, lengthening the exposure duration, dosage level manipulation, and the inclusion of other parameters such as tissues, bone or serum mineral concentration would be worth understanding the exact underlying mechanisms induced by dietary fumonisins.
2. The negligible but obvious abundance of *Lactobacillus sp*. opens the possibility for further research into the detoxification of dietary fumonisins by certain *Lactobacilli sp.*
3. The current results have emulated a situation in which fumonisins can result in unexplained production losses due to unclear clinical signs, although such doses are unusual in a commercial setting. The findings of this study should serve as a reminder of the value of continual animal feed evaluation in the animal production industry, particularly in light of the ongoing extreme weather events that are absolutely important factors in the development of mycotoxins.

## 6. New Scientific Results

1. Dietary fumonisins exposure at 15 or 30 mg/kg diet concentration depressed the apparent total tract digestibility of Ca and P, but not the Ca and P retention in nursery pigs in a not significant dose-dependent manner.
2. The retention of Cu and Zn in nursery pigs is impaired in case of FUMs intoxication, which was attributed completely to the reduction in the digestibility of these two minerals.
3. Using amplicon sequencing of the 16 rRNA gene V3-V4 regions and the QIIME 1.9.0 pipeline, it was established that dietary dose of either 15 or 30 mg/kg FUMs fed to nursery pigs resulted in a significant down-growth of some beneficial bacteria such as *Alloprevetolla, Campylobacter* and *Lachnospiraceae: Incertae Sedis* (in the duodenum), *Turicibacter* (in the jejunum) and *Clostridium sensu stricto 1* (in the ileum).
4. The faecal populations were shifted to the abundance of gut health promoting families such as Ruminococcaceae and Lachnospiraceae and their respective genera *Solobacterium, Faecalibacterium, Anaerofilum, Ruminococcus, Subdoligranulum, Pseudobutyrivibrio, Coprococcus,* and *Roseburia*.
5. Based on the observed abundance trends, *Lactobacillus sp*. present in the duodenum, ileum, and jejunum displayed a typical dose-response increment pattern, with the highest abundance in the duodenum and a decreasing trend in the distal direction of the intestine.
6. Dietary fumonisins in a concentration of 15 or 30 mg/kg diet did not affect the caecal fermentation outputs in nursery pigs.
7. Dietary dose of 40 mg/kg FUMs in 7- or 21-days exposure interval had a significant effect on the apparent ileal digestibility of arginine, histidine, and tyrosine. Although the permissible level of fumonisins is 5 mg/kg in swine feeds, this result indicates that gut functioning, at least the absorption of dietary crude protein and amino acids, is not impaired by a relatively high dose of fumonisins in fattening pigs.

## 7. References


Association of Analytical Chemists (AOAC) (2000): Official Methods of Analysis. 17th Edition, Gaithersburg, MD, USA.

Botsoglou, N. A., Fletouris, D. J., Papageorgiou, G. E., Vassilopoulos, V. N., Mantis, A. J. and Trakatellis, A. G. (1994): Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *Journal of Agricultural and Food Chemistry*, *42*(9), 1931–1937.

Bushnell, B., Rood, J. and Singer, E. (2017): BBMerge – Accurate paired shotgun read merging via overlap. *PLoS ONE*, *12*(10), 1–15. https://doi.org/10.1371/journal.pone.0185056

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K. and Gordon, J. I. (2010): QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336.

Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L. and Chen, F. (2017): The maturing development of gut microbiota in commercial piglets during the weaning transition. *Frontiers in Microbiology*, *8*, 1–13. https://doi.org/10.3389/fmicb.2017.01688

Fodor, J., Kametier, L. and Kovács, M. (2006): Practical aspects of fumonisin production under laboratory conditions. *Mycotoxin Research*, *22*(4), 211–216. https://doi.org/10.1007/BF02946744

Hazra, A. and Gogtay, N. (2016): Biostatistics series module 3: Comparing groups: Numerical variables. *Indian Journal of Dermatology*, *61*(3), 251–260. https://doi.org/10.4103/0019-5154.182416

Ju, F. and Zhang, T. (2015): 16S rRNA gene high-throughput sequencing data mining of microbial diversity and interactions. *Applied Microbiology and Biotechnology*, *99*(10), 4119–4129. https://doi.org/10.1007/s00253-015-6536-y

Lawrence, R. A. and Burk, R. F. (1978): Species, tissue and subcellular distribution of non Se-dependent glutathione peroxidase activity. *The Journal of Nutrition*, *108*(2), 211–215. https://doi.org/10.1093/jn/108.2.211

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H. and Robinson, C. J. (2009): Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541.

Sedlak, J. and Lindsay, R. H. (1968): Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry*, *25*, 192–205.

SPSS. (2012): SPSS for Windows version 20. IBM SPSS statistics for Windows. Armonk (NY): IBM Corp.

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J. R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E. R. and Knight, R. (2017): Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, *5*(1), 1–18. https://doi.org/10.1186/s40168-017-0237-y

Xia, Y. and Sun, J. (2017): Hypothesis testing and statistical analysis of microbiome. *Genes and Diseases*, *4*(3), 138–148. https://doi.org/10.1016/j.gendis.2017.06.001

## 8. Publications and Presentations

List of publications relevant to the research


1. Zeebone, Y.Y., Kovács, M., Bóta, B. and Halas, V. (2022): The effect of dietary fumonisin exposure on apparent ileal digestibility of amino acids in fattening pigs. *Agriculture, 12*(10), 1720. https://doi.org/10.3390/agriculture12101720
2. Zeebone, Y. Y., Kovács, M., Bóta, B., Zdeněk, V., Taubner, T. and Halas, V. (2022): Dietary fumonisin may compromise the nutritive value of feed and distort copper and zinc digestibility and retention in weaned piglets. *Journal of Animal Physiology and Animal Nutrition*, 1–14. https://doi.org/10.1111/jpn.13724
3. Zeebone, Y.Y., Kovács, M., Bota, B., Balogh, K. and Halas, V. (2022): P164. Dietary fumonisins potentially modulate some health indicators in weaned piglets. *Animal Science Proceedings, 13*(2), 208.
4. Zeebone, Y.Y., Kovács, M., Bóta, B. and Halas, V. (2020): Effects of dietary fumonisins on nutrients digestibility in weanling pigs. *Acta Fytotechnica et Zootechnica, 23*, 23–28. https://doi.org/10.15414/afz.2020.23.mi-fpap.23-28
5. Zeebone, Y.Y., Kovács, M. and Halas, V. (2020): Effects of fumonisin $B_1$ on the gastrointestinal tract functionality. A review. (A fumonizin B1 hatása a tápcsatorna működésére. Irodalmi áttekintés). *Állattenyésztés és Takarmányozás, 69*(1), 53–65.


List of other publications


6. Kócsó, D.J., Ali, O., Kovács, M., Mézes, M., Balogh, K., Kachlek, M.L., Bóta, B., Zeebone, Y.Y. and Szabó, A. (2021): A preliminary study on changes in heat shock protein 70 levels induced by *Fusarium* mycotoxins in rats: *in vivo* study. *Mycotoxin Research, 37*(2), 141–148. https://doi.org/10.1007/s12550-021-00425-z
7. Szabó, A., Szabó-Fodor, J., Kachlek, M., Mézes, M., Balogh, K., Glávits, R., Ali, O., Zeebone, Y.Y. and Kovács, M. (2018): Dose and exposure time- dependent renal and hepatic effects of intraperitoneally administered fumonisin $B_1$ in rats. *Toxins, 10*(11), 465. https://doi.org/10.3390/toxins10110465
8. Boateng, M., Okai, D.B., Frimpong, Y.O. and Zeebone, Y.Y. (2015): Wet brewers' spent grains and wet brewers' spent yeast: problems associated with their usage and suggested

solutions: a case study of the Ejisu-Juaben Municipality of Ghana. *Age, 2*(8), 6. Retrieved January 5, 2023, from http://www.lrrd.org/lrrd27/1/boat27005.htm

Oral presentations

1. Zeebone Y.Y., Kovács, M., Bóta, B. and Halas, V. **Effect of dietary fumonisin exposure on apparent ileal digestibility of amino acids in fattening pigs**. The 29th Animal Science Days International Symposium, September 13–17, 2021, Gödöllő, Hungary.
2. Zeebone, Y.Y., Kovács, M., Bóta, B. and Halas, V. **Effects of dietary fumonisins on nutrients digestibility in weanling pigs**. The 28th Animal Science Days International Symposium, September 23–25, 2020, Padova, Italy.
3. Zeebone Y.Y., Kovács, M. and Szabo-Fodor, J. **Dose dependent oxidative stress-mediated toxicity of fumonisin $B_1$ in rats**. The Scientific Student Conference in Applied Life Sciences, November 21, 2018, Faculty of Agricultural and Environmental Sciences, Kaposvári Egyetem, Kaposvár, Hungary.

Poster presentations

4. Zeebone, Y.Y., Kovács, M., Bóta, B., Libisch, B., Olasz, F. and Halas, V. **Potential effect of dietary fumonisins on the colonization of *Lactobacilli* in the gut and fecal microbiota of weaned pigs.** Hungarian University of Agriculture and Life Sciences 20th International Symposium on Animal Nutrition, KÁN University Days, September 29, 2022, Kaposvár, Hungary.
5. Zeebone, Y.Y., Kovács, M., Bota, B., Balogh, K. and Halas, V., 2022. **Dietary fumonisins potentially modulate some health indicators in weaned piglets**. The 15th International Symposium on Digestive Physiology of Pigs, May 17–20, 2022, Rotterdam, The Netherlands.