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The Effect of Dietary Fumonisin Exposure on Apparent Ileal Digestibility of Amino Acids in Fattening Pigs

Yarsmin Yunus Zeebone [1,2,*], Melinda Kovács [1,2], Brigitta Bóta [2] and Veronika Halas [3]

[1] Agribiotechnology and Precision Breeding for Food Security National Laboratory, Department of Physiology and Animal Health, Institute of Animal Physiology and Nutrition, Hungarian University of Agriculture and Life Sciences-Kaposvár Campus, 40, Guba S. Str., H-7400 Kaposvár, Hungary
[2] ELKH-MATE Mycotoxins in the Food Chain Research Group, 40, Guba S. Str., H-7400 Kaposvár, Hungary
[3] Department of Farm Animal Nutrition, Institute of Animal Physiology and Nutrition, Hungarian University of Agriculture and Life Sciences Kaposvár Campus, 40, Guba S. Str., H-7400 Kaposvár, Hungary
* Correspondence: yarsminyunuszee3@gmail.com; Tel.: +36-204-105-176

Abstract: The cellular toxicity of the *Fusarium* mycotoxin fumonisins (FUMs) has been widely accounted for. However, the ability of FUMs to destroy intestinal functions is an emergence of growing concern. Thus, this experiment ascertained whether dietary FUMs obstruct the apparent ileal digestibility (AID) of crude protein (CP) and amino acids (AAs) in fattening pigs during either short (7 d)- or long (21 d)-term exposure. Ten Danbred fattening pigs (initial body weight (BW) of 67.5 ± 4.1) inserted with a post-valve T-cecum cannula in the terminal ileum were enrolled in the trial. The pigs were randomly divided into a control group fed a basal commercial diet and a group fed in vitro-produced FUMs to provide a 40 mg FUMs/kg-contaminated diet. Titanium dioxide was added at an inclusion rate of 0.5% as an indigestible marker to diets. During two separate periods, ileal digesta were collected for 3 consecutive days for the determination of the AID of CP and the various dispensable and indispensable AAs. Data were subjected to two-way ANOVA of SPSS version 20.0 software using FUMs dose (i = 2; 0 or 40 mg FUMs/ kg feed) and duration (j = 2; short- vs. long-term exposure) as fixed factors. According to our findings, a dietary intake of 40 mg/kg FUMs substantially interfered with the AID of arginine, histidine, and tyrosine (p = 0.003, 0.047, and 0.047, respectively) in terms of the dose and duration interaction effect. In addition, the main duration effect of the AID of histidine was significant (p < 0.001). It is, therefore, conceivable that a dietary dose of a 40 mg/kg FUMs-contaminated diet does not drastically affect CP and AAs digestibility in fattening pigs over a period of 7 or 21 days.

Keywords: amino acids; apparent ileal digestibility; crude protein; fattening pigs; fumonisins; mycotoxins

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Citation: Zeebone, Y.Y.; Kovács, M.; Bóta, B.; Halas, V. The Effect of Dietary Fumonisin Exposure on Apparent Ileal Digestibility of Amino Acids in Fattening Pigs. *Agriculture* **2022**, *12*, 1720. https://doi.org/10.3390/agriculture12101720

Academic Editor: Łukasz Jarosz

Received: 8 September 2022
Accepted: 17 October 2022
Published: 19 October 2022

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1. Introduction

Fumonisins (FUMs) are a type of *Fusarium* mycotoxin that particularly occur in corn and its related products. Although several congeners of FUMs have been discovered, FB_1 is of utmost toxicological concern, followed by the second, FB_2, and then the least toxic, FB_3 [1,2]. Depending on animal species, FB_1 toxicity can range from equine leukoencephalomalacia (ELEM) in horses [3] to nephrotoxicity in rabbits, rats, and lambs, as well as hepatotoxicity in all examined species [4]. In pigs, porcine pulmonary edema (PPE) [5] and cardiovascular toxicity [6] have been reported. Due to its structural resemblance to the sphingoid bases sphingosine and sphinganine, FB_1 remains capable of interfering with sphingolipid metabolism, which subsequently leads to disturbances in cell differentiation, proliferation, and apoptosis [7].

Monogastric and younger animals are often more susceptible to the negative effects of mycotoxins than ruminants and older animals. The induction of the toxic pathways of the various mycotoxins is dependent on the duration of exposure, chemical constituent,

concentration, species, sex, age, and sensitivity of the infected animal. Additionally, long-term exposure to low concentrations of mycotoxins may result in production losses and raise the risk and prevalence of various diseases [8]. Furthermore, diminishing the nutritive value of the mixed feed by different mycotoxins has been proven by some studies [9,10]. Pigs and poultry are the most vulnerable to the effects of mycotoxins among the monogastric species of animals. As a result, these species have been used in the bulk of mycotoxin toxicity research [11]. In practice, the frequently high proportion of corn in their diets as the main energy source is a clear indication of pigs' susceptibility to FUMs intoxication. Accordingly, the European Commission permitted levels of FUMs (FB_1 + FB_2) for corn in finished swine feed to be set to 5 mg/kg [12].

The gastrointestinal tract (GIT) epithelium is the first barrier to come into contact with mycotoxins. Fumonisins are poorly absorbed, so the GIT is a highly exposed organ. According to our hypothesis, a high FUMs load on gut epithelial cells may alter sphingolipid metabolism. A decrease in complex sphingolipids may, therefore, result in reduced intestinal barrier function [13]. Immune disturbances [14] and compromised barrier integrity and functions [15] have been linked with FUMs exposure at all age growths of pigs. Furthermore, some recent findings suggest that exposure to chronic FUMs intoxication (6 mg/kg) induces changes in intestinal villi morphology in young pigs [16], which may have a consequence on the digestibility of nutrients, as confirmed in a study with broilers [17]. As mentioned earlier, mostly young animals such as broiler chickens or nursery pigs are used in mycotoxin studies. However, it is an open question whether protein digestibility is compromised by FUMs in fattening pigs in a less sensitive phase such as in fatteners.

To the best of our knowledge, the effect of FUMs on the apparent ileal digestibility (AID) of amino acids (AAs) in pigs has not been studied. We anticipated in the current investigation that at a dietary level of 40 mg/kg total FUMs (FB_1 + FB_2 + FB_3) fed to fattening pigs, the adverse effects on digestibility of AAs could be more severe during a longer exposure time (21 days) than for a shorter exposure time (7 days).

2. Materials and Methods

The research protocol was reviewed and authorized by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority, Hungary (SOI/31/00997-7/2018).

2.1. Experimental Design, Conditions, and Diet Preparation

Ten Danbred breed fattening pigs (average bodyweight: 65.5 ± 4.1 kg) fitted with a post-valve T-cecum (PVTC) cannula as described by van Leeuwen et al. [18] were enrolled in the study. For the purpose of the collection of ileal digesta samples for the digestibility study, the pigs were kept in individual crates (200 × 120 cm) located in the Experimental Animal Unit of the Department of Animal Nutrition (MATE-KC). A commercial feed comprising corn, barley, wheat, extracted soybean mean, limestone, monocalcium phosphate, sodium chloride, and additives was used as the basal diet. The proximate analysis of the feed is shown in Table 1. The pigs were randomly assigned to 2 different diets: a control diet that contained no FUMs 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 × 2 factorial design, the independent variables were the treatments (control or 40 mg/kg FUMs intoxication), and the exposure time [(7 days (short-term exposure) or 21 days (long-term 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.

Table 1. Feed composition and analyzed proximate composition of feed.

Component, g/kg	
Corn	612.35
Soybean meal	247
Barley	101
Sunflower oil	40
Limestone	13
Monocalcium phosphate	11.1
Vitamin and mineral premix	5.0
Salt	4.0
L-Lysine HCl	1.3
DL-methionine	1.0
L-threonine	0.25
Item, g/kg	
Crude protein	167
Crude fat	32
Crude fiber	23
Starch	479
Crude ash	51
Calcium	7.14
Phosphorus	5.37
Sodium	1.81

Preparation of FUMs used for the pigs followed a modified description of Fodor et al. [19] using a *Fusarium verticillioides* fungal culture. The final fungal culture concentrations were 25.57 mg/g FB_1, 6.17 mg/g FB_2, and 3.01 mg/g FB_3 (Table 2). The finely ground culture was mixed carefully and thoroughly into the ration of the experimental animals to provide a daily FUMs (FB_1 + FB_2 + FB_3) feed concentration of the 40 mg/kg diet. In the in vitro-produced FUMs, 730 g of it was added first to 5 kg of feed and mixed by hand, and then those premixes were further added to each 400 kg feedlot and homogenized in a feed mixer. The mycotoxin concentration of the control and the experimental feed was then determined with the LC-MS method, which utilized a Shimadzu Prominence ultra-fast liquid chromatography (UFLC) separation system equipped with an LC-MS-2020 single-quadrupole (ultra-fast) liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) with an electrospray source. The limit of detection (LOD) for FB_1 and FB_2 were 0.031 and 0.051 mg/kg, respectively; LOD for FB_3 was not measured. Diet fed to the control group did not contain detectable amounts of FUMs or other mycotoxins [the full absence of zearalenone (ZEN), deoxynivalenol (DON), and T-2 toxin was confirmed] (Table 2).

Table 2. Level of in vitro-produced (fungal culture) total fumonisins used for the formulation of contaminated diet, dietary level and limit of detection of total fumonisins in experimental feed.

Mycotoxins	**LOD, mg/kg**	**Fungal Culture, mg/g**	**Control (No Contamination)**	**40 mg/kg FUMs, mg/kg**
FB_1	0.031	25.57	nd	30.37
FB_2	0.051	6.17	nd	7.12
FB_3	-	3.01	nd	3.1
Zearalenone	0.005	-	nd	-
Deoxynivalenol	0.053	-	nd	-
T-2	0.011	-	nd	-

FB_1 = fumonisin B1; FB_2 = fumonisin B2; FB_3 = fumonisin B3; FUMs = fumonisins; LOD = Limit of detection; nd = not detected.

Titanium dioxide (0.5%) was added to the feed as an indigestible marker to evaluate the AID of CP and AAs. Feed was offered at an amount that covers 2.8 times the maintenance energy requirement [20] and was provided twice a day in two equal portions. Drinking water was made available ad libitum.

2.2. Digestibility Trial

The pigs were cannulated according to a method by [18]. In brief, the caecum is removed, and a large T-cannula is used in its stead. After the caecum is cut, the flange of the cannula is introduced in the large intestine, and the cannula aperture is positioned in front of the ileocaecal valve. The flange is fixed to the intestinal wall by tightening a preplaced purse-string suture. The cannula is then exteriorized through an incision in the body wall, fixed externally by mounting a ring of silicone rubber, and closed with a silicone rubber stop. The digesta from the ileum flows into the colon after the cannula is closed. When the ileocaecal valve is open, it protrudes into the cannula's aperture, allowing the digesta to flow straight into it and be collected using plastic bags [18]. 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 the 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 of ileal digesta began and was labeled 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 ileal digesta collection began and labeled the long-term exposure period.

Collection of the digesta samples from each animal was a matter of constantly checking whether the plastic bags were filled with enough ileal digesta, i.e., not too small, and not too much that could spill out of the bags. The collected ileal digesta samples were weighed and immediately stored at −20 °C to prevent bacterial degradation of the amino acids. At the end of the experiment, the samples were freeze-dried and ground before the analyses.

Pigs were euthanized by exsanguination after sedation (intramuscular injection of zolazepam and tiletamine (2.5 mg/kg, Zoletil, Virbac)), xylazine (3 mg/kg, CP-Xylazin 2%, CP-Pharma Handelsges), and azaperone (6 mg/kg, Stresnil, Janssen-Cilag) at the end of the experiment.

2.3. Laboratory Analysis

Chemical analysis of samples was performed in cooperation with the laboratory of the MATE-KC. The AAs—aspartic, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, and ammonia—were measured with an automatic analyzer according to the Hungarian MSZ EN ISO 13903:2005 standardization procedure. Nitrogen was determined with the Kjeldahl method [21]. Table 3 shows the analyzed AAs in the experimental feed.

Titanium dioxide (TiO_2) was analyzed by measuring 150 mg of sample and a Foss Se + K_2SO_4 catalyst pill in a digestion tube, after which 25 mL sulfuric acid and 6 mL H_2O_2 were added. The mixture was digested at 400 °C for 4 h. After cooling, the mixture was transferred to a 100 mL volumetric flask and was filled with water. A 10 mL volume of the sample was transferred in a test tube, and 1 mL of color reagent (mixture of sulfuric acid, phosphoric acid, and hydrogen peroxide) was added and was left to stand for one hour. It was measured with a spectrophotometer at a 410 nm wavelength and was compared to a standard curve of different dilutions of TiO_2 prepared similarly (the lab's own method).

2.4. Calculation and Statistical Analysis

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

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

where AID is the apparent ileal digestibility, TiO_2 is titanium dioxide, CP is the crude protein, and AA is the amino acid in question.

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

Table 3. Analyzed amino acids in experimental feed for cannulated-fattening pigs.

Amino Acids, g/100 g *Sample*	
Aspartic acid	1.65
Threonine	0.66
Serine	0.84
Glutamic acid	3.33
Proline	1.12
Glycine	0.69
Alanine	0.89
Cysteine	0.26
Valine	0.76
Methionine	0.28
Isoleucine	0.65
Leucine	1.43
Tyrosine	0.45
Phenylalanine	0.8
Histidine	0.42
Lysine	0.95
Ammonia	0.3
Arginine	0.96

3. Results

Results showed no significant differences in the initial and final body weights (BW) of both groups of pigs (Table 4). No difference in the AID values were detectable if the effect of the two exposure times was examined separately. The main effect of duration (d) tended to impact the AID of only histidine (Table 5). Dose and duration interaction wise, results showed that a long exposure time of 40 mg/kg dietary FUMs lowered the AID of tyrosine and arginine compared to a shorter time, while the opposite was observed for the control group. Meanwhile, the AID of histidine for both control and FUMs-fed groups showed high AID values when we compare the durations of 7 d and 21 d. Although the increment observed in the AID values for the control was considerably higher than the FUMs-fed group (Table 5).

Table 4. Effect of dietary fumonisins on the growth rate of fattening pigs (data are means ± standard deviation (SD) of 5 individuals/group).

Item, Kg	**Control**	**40 mg/kg FUMs**
Initial BW	67.3 ± 5.0	67.8 ± 3.7
Final BW	81.2 ± 5.7	83.1 ± 5.7

BW = bodyweight. Absence of lowercase letters denotes no significant difference at $p < 0.05$.

Table 5. Effect of short (7 d)- or long (21 d)-term exposure of dietary fumonisins on apparent ileal digestibility of dry matter, crude protein, and amino acids in fattening pigs (data are means ± standard deviation (SD) of 5 individuals/group).

Duration, d	**Short-Term Effect, 7 d**		**Long-Term Effect, 21 d**		***p*-Values**		
Treatments, t	**Control**	**40 mg/kg FUMs**	**Control**	**40 mg/kg FUMs**	**Treatment (t) Effect**	**Time (d) Effect**	**Interaction (t × 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
Indispensable 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
Dispensable amino acids							
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	0.42	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. Discussion

Dietary AAs are required by animals primarily for maintenance and protein accretion. Because protein is the most expensive component among feed additives, to produce economical and high-quality products, the livestock industry is dependent on the optimal use of dietary AAs [22]. The best possible use is also crucial for animal health because it may prevent liver and kidney damage and also save the environment by reducing nitrogen emissions. When it comes to nutrient absorption, immunological response, and growth efficiency, AAs are crucial metabolic intermediaries [23]. Further, dietary AAs generated by animal cells perform regulatory functions in nutrient metabolism such as protein turnover and lipid synthesis and oxidation to promote lean tissue development and adipose tissue decrease [24]. These processes also tend to be major targets for mycotoxins' harmful effects. Looking at the unanticipated ways FUMs have emerged to induce toxic insults, the present study examined the potentiality of FUMs to impede ileal digestibility of CP and AAs in fattening pigs.

As demonstrated by the findings of the present study, dietary FUMs did not obstruct the AID of CP regardless of the exposure time. Elsewhere, when an increasing dose of dietary FB_1 (0.2, 5.0, 10, and 15 mg/kg) was fed to growing pigs in a 6-month trial, a noticeable reduction in crude fat and CP digestibility in a dose-response manner was highlighted [25]. Moreover, in a subchronic investigation, a substantial drop in digestibility values of CP was confirmed in Wistar rats subjected to increasing amounts of FB_1 (0.2, 10, or 20 mg/kg diet), albeit a detrimental effect on growth performance was accompanied [26]. In a much longer exposure feeding trial (6 months) consisting of a 10.0 or 15.0 mg FB_1/kg-contaminated diet, lower serum protein values were seen in pubertal boars, which were subsequently attributed to an impairment of the protein metabolism in the pigs [27]. Although in this study there was no digestibility measurement, it was hypothesized that the exposure to the FB_1 diet over such a long period might have interfered with some physiological processes of digestion and absorption and thus, resulted in the inefficient use of dietary protein since protein synthesis is closely linked to the availability of dietary protein [28].

Arginine is a central intestinal metabolite, both as a constituent of protein synthesis, and together with threonine, glutamine, methionine, and cysteine, plays a critical role in protecting gut barrier function and maintaining gut mucosal immunity. This study showed that the AID of arginine was reduced over a period of 21 days as opposed to 7 days in the FUMs-infected group as opposed to the control group. Fumonisin B1 is known to be capable of mildly triggering oxidative damage or apoptosis depending on the species and cell types [29]. Nitric oxide (NO) is known to play a crucial function in controlling the antioxidant defense system [30]. In the small intestine, arginine promotes the generation of NO within physiological limits [31]. Accordingly, it has been hypothesized that a 1% supplementation of arginine aids in scavenging the excess reactive oxygen species (ROS) brought on by mycotoxin-contaminated feed, improving the balance between the production of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical and the biological defense against the toxicity of these oxidants in growing pigs [32]. We could plausibly link the oxidative damage propensity of FUMs to the poor AID of arginine. To substantiate this supposition, however, more research is needed. Histidine and tyrosine, in contrast to arginine, are AAs that are rarely examined in investigations of intestinal diseases [33]. As a result, with more investigation into the molecular and physiological workings that control the actions of such AAs, we will be able to make reliable judgments about how mycotoxins affect the digestibility of these AAs in the GIT.

To our knowledge, there have not been works highlighting the effects of solely FUMs in AAs digestibility. However, a decent number of similar works have been conducted but as co-contamination trials of chiefly *Fusarium* mycotoxins, including FUMs. Co-contamination of food/feed commodities with mycotoxins is increasingly becoming difficult to fully anticipate the detrimental effects of the combined toxicity of these toxins, with the majority of mycotoxin mixes having additive, antagonistic, or synergistic effects [34]. Thus, it may aggravate the negative effects of mycotoxins and pose serious health risks to animals. In a study carried out by Jo and colleagues [35] with the *Fusarium* mycotoxins DON and ZEN, the authors used equal levels, i.e., 10 mg/kg of DON and ZEN, to investigate their effects on the AID of CP and AAs in growing pigs. The study revealed a significant reduction in digestibility of lysine, threonine, valine, and tryptophan caused by DON, whereas ZEN effects were unremarkable [35]. Although unclear about the results, the authors of the study suggested this to be a result of DON's popular ability to disturb intestinal processes, and so, digestion and absorption of dietary components are negatively impacted in the process.

In a similar co-contamination trial, young pigs were exposed to a combination of aflatoxin (AFB1) (0.62 ppb), ochratoxin (11.39 ppb), DON (3 ppm), and FB_1 (2 ppm) to assess the glutamate effect on ameliorating perturbation to the intestinal structure of the pigs. The authors found suppression of growth, impaired intestinal architecture, oxidative damage, and modification of the serum AA profile in pigs that received the intoxicated diet compared to the toxin-free group [32]. Previous research has also shown that adding the combination of DON and FUMs (DON 5.0 mg/kg + FUMs 20 mg/kg) to the diets of poultry for 21 days resulted in a significant decrease in DM and ileal energy digestibility. DON, FUMs, or their combination, on the other hand, showed no effect on endogenous AAs loss or standardized CP and AAs digestibility [36], which slightly agrees with the outcome of the present study.

5. Conclusions

In conclusion, the results show that 40 mg/kg FUMs had no drastic effect on AA digestibility, while the permissible level of 5 mg/kg in swine feeds indicates that gut functioning—at least the absorption of dietary CP and AAs—is not impaired by a relatively high dose of FUMs in fattening pigs.

Author Contributions: Conceptualization, M.K. and V.H.; methodology, M.K. and V.H.; validation, M.K. and V.H.; formal analysis, Y.Y.Z. and V.H.; writing—original draft preparation, Y.Y.Z.; writing—review and editing, Y.Y.Z. and V.H.; visualization, V.H.; experiment supervision, B.B.; funding acquisition, M.K. and V.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Innovation and Technology (GINOP-2.3.2-15-2016-00046) and the Ministry of Human Resources (EFOP-3.6.3-VEKOP-16-2017-00005) program, the ELKH TKI office for Supported Research Groups and the One Health (2020-4.1.1-TKP2020) project of the National Research Development and Innovation Office.

Institutional Review Board Statement: The research protocol was reviewed and authorized by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority, Hungary (SOI/31/00659-14/2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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